Published in final edited form as:
Plant Mol Biol. 2006 July ; 61(4-5): 781–793. doi:10.1007/s11103-006-0049-0.

Floral patterning defects induced by *Arabidopsis APETALA2* and microRNA172 expression in *Nicotiana benthamiana*

Sizolwenkosi Mlotshwa
Department of Botany & Plant Sciences, University of California, Riverside, California 92521, USA

Department of Biological Sciences, University of South Carolina, Columbia, South Carolina 29208, USA

Zhiyong Yang
Department of Botany & Plant Sciences, University of California, Riverside, California 92521, USA

YunJu Kim
Department of Botany & Plant Sciences, University of California, Riverside, California 92521, USA

Xuemei Chen
Department of Botany & Plant Sciences, University of California, Riverside, California 92521, USA

Abstract

Floral patterning and morphogenesis are controlled by many transcription factors including floral homeotic proteins, by which floral organ identity is determined. Recent studies have uncovered widespread regulation of transcription factors by microRNAs (miRNAs), ~21-nucleotide non-coding RNAs that regulate protein-coding RNAs through transcript cleavage and/or translational inhibition. The regulation of the floral homeotic gene *APETALA2* (*AP2*) by miR172 is crucial for normal *Arabidopsis* flower development and is likely to be conserved across plant species. Here we probe the activity of the *AP2*/miR172 regulatory circuit in a heterologous *Solanaceae* species, *Nicotiana benthamiana*. We generated transgenic *N. benthamiana* lines expressing *Arabidopsis* wild type *AP2* (*35S::AP2*), miR172-resistant *AP2* mutant (*35S::AP2m3*) and *MIR172a-1* (*35S::MIR172*) under the control of the cauliflower mosaic virus 35S promoter. *35S::AP2m3* plants accumulated high levels of *AP2* mRNA and protein and exhibited floral patterning defects that included proliferation of numerous petals, stamens and carpels indicating loss of floral determinacy. On the other hand, nearly all *35S::AP2* plants accumulated barely detectable levels of *AP2* mRNA or protein and were essentially non-phenotypic. Overall, the data indicated that expression of the wild type *Arabidopsis AP2* transgene was repressed at the mRNA level by an endogenous *N. benthamiana* miR172 homologue that could be detected using Arabidopsis miR172 probe. Interestingly, *35S::MIR172* plants had sepal-to-petal transformations and/or more sepals and petals, suggesting interference with *N. benthamiana* normal floral homeotic gene function in perianth organs. Our studies uncover the potential utility of the *Arabidopsis AP2*/miR172 system as a tool for manipulation of floral architecture and flowering time in non-model plants.

xuemei.chen@ucr.edu.

Keywords

APETALA2; *Arabidopsis*; microRNA; miR172; *Nicotiana benthamiana*

Introduction

The genetic control of floral patterning has been well studied in *Arabidopsis thaliana*. The *Arabidopsis* flower consists of four sepals, four petals, six stamens and two fused carpels from the outer to inner whorls. Floral organ identities are specified by transcription factors encoded by 3 classes of floral homeotic genes: the A, B and C function genes (Coen and Meyerowitz 1991; Meyerowitz et al. 1991; reviewed in Gutierrez-Cortines and Davies 2000; Lohmann and Weigel 2002; Jack 2004; Krizek and Fletcher 2005). Class A genes specify sepals and interact with B genes to specify petals; C function genes specify carpels and interact with B function genes to specify stamens. A and C function genes act antagonistically to restrict each other's activities to perianth and reproductive organs respectively, a phenomenon that is well studied for *Arabidopsis APETALA2* (*AP2*), a class A gene, and *Arabidopsis AGAMOUS* (*AG*), a class C gene (Kunst et al. 1989; Bowman et al. 1991; Drews et al. 1991; Bomblies et al. 1999; Mizukami and Ma 1997; Krizek et al. 2000).

Recent studies have uncovered the role of microRNAs (miRNAs), ~21-nucleotide non-coding RNAs, as regulators of floral patterning gene expression in *Arabidopsis* flower development (Aukerman and Sakai 2003; Schmid et al. 2003; Achard et al. 2004; Chen 2004; Mallory et al. 2004a; Baker et al. 2005; Millar and Gubler 2005). Plant miRNAs regulate cognate protein coding RNAs through either transcript cleavage or translational inhibition or both (reviewed in Bartel and Bartel 2003; Carrington and Ambros 2003; Bartel 2004; Dugas and Bartel 2004; He and Hannon 2004; Mallory and Vaucheret 2004; Kidner and Martienssen 2005; Chen 2005; Valencia-Sanchez et al. 2006). The translational inhibition of *AP2* expression by miR172 is crucial for normal floral patterning (Aukerman and Sakai 2003; Chen 2004). Over expression of miR172-resistant *AP2* in *Arabidopsis* gave elevated AP2 protein levels and led to an indeterminate floral meristem that produces numerous stamens or petals (Chen 2004). On the other hand, over expression of miR172 was found to reduce AP2 protein levels resulting in phenotypes reminiscent of *ap2* mutants (Aukerman and Sakai 2003; Chen 2004). In maize, the levels of a miR172 homologue during vegetative growth are inversely correlated with the transcript levels of its *AP2*-like target gene, *glossy15*, suggesting that miR172 promotes vegetative phase changes by reducing *glossy15* expression through transcript cleavage (Lauter et al. 2005). These findings demonstrate the importance of the miR172/AP2 regulatory control in plant development.

AP2-like genes containing putative miR172 target sites are conserved in eudicots, monocots and early land plants (Aukerman and Sakai 2003; Axtell and Bartel 2005). It is thus conceivable that transgenic expression of *Arabidopsis* miR172 and *AP2* in heterologous angiosperm species would elicit similar developmental phenotypes as those previously uncovered in *Arabidopsis* (Aukerman and Sakai 2003; Chen 2004). If true, this will be a potent genetic engineering tool for manipulation of floral architecture in diverse plant species. To test this hypothesis, we generated transgenic *Nicotiana benthamiana* lines expressing *Arabidopsis* miR172, and wild type and miR172-resistant *AP2* genes. Indeed, expression of *Arabidopsis AP2* in *N. benthamiana* resulted in floral alterations similar to those in *Arabidopsis*. However, expression of *Arabidopsis* miR172 in *N. benthamiana* resulted in floral homeotic transformations that implicate divergent functions of *N. benthamiana AP2* from *Arabidopsis AP2*.

Materials and methods

DNA constructs and bacterial strains

Plant transformation binary constructs containing *Arabidopsis AP2* and *MIR172* genes under control of the cauliflower mosaic virus (CaMV) double 35S promoter in the vector pMAT137hm were described previously (Chen 2004). The constructs were used for *N. benthamiana* transformation in this study. They include wild type *AP2* cDNA (35S::*AP2*), mutant *AP2* cDNA with six mismatches to miR172 that do not change the amino acid sequence (*35S::AP2m3*) and *MIR172a-1* (*35S::MIR172*) in *Agrobacterium tumefaciens* strain ASE.

Plant transformation and growth conditions

N. benthamiana leaf explants were prepared from *in vitro* grown sterile plants and transformed using *A. tumefaciens* mediated transformation as described previously (Mlotshwa 2000; Mlotshwa et al. 2002). The plants were regenerated in a plant growth chamber at 24°C under 16 h-light/8 h-dark cycles. Kanamycin resistance was used as a selection marker for stable transformants. Following root induction, plants were transferred to soil in the green house and grown at 25°C under 16 h-light/8 h-dark cycles.

Plant genomic DNA isolation and gel blot analysis

Plant genomic DNA was isolated from transgenic lines using the Qiagen DNeasy plant mini kit according to instructions in the accompanying protocol. 10 μg of DNA was digested with *Eco*RI, resolved by agarose gel electrophoresis and blotted onto hybond membranes (Amersham). The CaMV 35S promoter DNA fragment was randomly labeled with ^{32}P using the Ready-To-Go DNA labeling beads (Amersham) and hybridized to DNA blots at 65°C in hybridization buffer (0.5 M Na_2HPO4, pH 7.2; 7% SDS; 1 mM EDTA; 1· denatured ssDNA).

Total RNA isolation and gel blot analysis

Floral buds were ground in liquid nitrogen and a small part of the fine powder was saved for protein extraction. The remainder of the powder was vortexed in a 1:1 mixture of RNA extraction buffer (100 mM Tris–HCl, pH8.0; 100 mM LiCl; 10 mM EDTA; 1% SDS) and phenol. Two volumes of chloroform were added and the aqueous phase, recovered following centrifugation, was precipitated with an equal volume of 4 M LiCl/10 mM EDTA. The samples were centrifuged to pellet the high molecular weight (HMW) RNA. DNA was removed from the supernatant by precipitation with 10% polyethylene glycol 8000/0.5 M NaCl for 30 min on ice followed by centrifugation. Low molecular weight (LMW) RNA was recovered from the supernatant by ethanol precipitation.

For RNA blot analysis, 10 lg of HMW RNA was resolved on denaturing agarose gels and blotted onto hybond membranes (Amersham). For transgenic *AP2* mRNA probe, a full length *Arabidopsis AP2* cDNA PCR fragment was radiolabelled with ^{32}P as described for DNA gel blot analysis above. To make ^{32}P-labelled RNA probe to detect endogenous *NbAP2-like1* mRNA, a T7 promoter was PCR-incorporated into the 3′ 647 nucleotides of the *N. benthamiana AP2-like1* (*NbAP2-like1*) coding sequence, which was *in vitro*-transcribed with T7 RNA polymerase using Ambion MAXIscript kit. The probes were hybridized to RNA blots at 65°C in Ambion ULTRAhyb buffer.

For miR172 analysis, 10 μg of LMW RNA was resolved on 20% polyacrylamide/7 M urea denaturing gels and blotted onto hybond membranes (Amersham). An oligonucleotide antisense to *Arabidopsis* miR172 was end-labelled with ^{32}P using T4 polynucleotide kinase

(New England Biolabs) and hybridized to miRNA blots at 42°C in Ambion ULTRAhyb-oligo buffer.

RT-PCR analysis

N. benthamiana total RNA was treated with RNase-free DNase1 (Ambion) and 5 μg was used for first strand cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen) and oligodT or gene specific primers at 55°C.

A 401 bp *N. benthamiana WUSCHEL* cDNA (*NbWUS*) fragment was amplified by RT-PCR using primers LeWUS-F (tta ttg gtt tca gaa cca taa agc tcg tga) and LeWUS-R (gat gca gaa att tga gat gat gtt ctc ttc a) designed from sequences conserved between *Arabidopsis WUS* (GenBank # AJ012310; At2g17950), *Lycopersicon esculentum* (tomato) *WUS* (GenBank # AJ538329) and *Petunia hybrida WUS* (GenBank # AF481951). The forward and reverse sequences of the PCR product confirmed it to be bona fide *NbWUS* (GenBank # DQ437634). Primers NbWUS-F (ggt gtt tgg aga tct gtt gat gat c) and NbWUS-R (aag tgg gag agt ttc taa agc aga g) were designed from the sequence and used for semi-quantitative RT-PCR analysis of *NbWUS* transcript levels in transgenic lines.

A 404 bp *N. benthamiana AG* cDNA (*NbAG*) fragment was amplified by RT-PCR using primers NAG1-F (gaa aac aca acg aat cgt caa gtc act tt) and NAG1-R (gtg taa atc aat ttc cct ctt ctg cat gta) designed from sequences conserved between *Arabidopsis AG* (At4g18960) and *Nicotiana tabacum AG* (*NAG1*; GenBank # L23925). The fragment was sequenced from the sense and antisense strands (GenBank # DQ437633). Upon BLAST searches with the translated protein sequence, NAG1 and petunia AG were the two top hits, suggesting that the sequence is likely bona fide *NbAG*. Primers NbAG-F (gcc tat gaa tta tct gtg ctc tgt g) and NbAG-R (gta ctc aat ttc agc aaa cag cag c) were designed from the *NbAG* partial sequence and used for semi-quantitative RT-PCR analysis of *NbAG* transcript levels in transgenic lines.

For *N. benthamiana DEFICIENS* (*NbDEF*) semi-quantitative RT-PCR analysis, primers NbDEF-F (atg gct cgt ggg aag atc cag atc aa) and NbDEF-R (tca agc tag agc aaa agt agt gat atc) were designed from *N. tabacum DEF* sequence (GenBank # X96428) and amplified fragments were confirmed to be *NbDEF* through sequencing (GenBank # DQ437635). For *N. benthamiana GLOBOSA* (*NbGLO*) semi-quantitative RT-PCR analysis, primers NbGLO-F (atg gga aga gga aag ata gag atc aaa ag) and NbGLO-R (gct cct cct cca tac ttt gag tct tt) were designed from *N. tabacum GLO* sequence (GenBank # X67959) and amplified fragments were confirmed to be *NbGLO* through sequencing (GenBank # DQ437636).

To clone *N. benthamiana AP2-like* genes, a ~600 bp fragment was amplified using oligodT and primer miR172-F (gct gca gca tca tca gga ttc t) designed from the miR172 target sites on *Arabidopsis AP2* (At4g36920), *P. hybrida AP2* (GenBank # AF132001; Maes et al. 2001) and tomato *AP2* (TIGR # TC162117). From BLAST searches, the sequenced oligodT/miR172-F fragment turned out to be part of the GenBank sequence CK287095, which represents the 3′ portion of an *N. benthamiana* transcript. CK287095 contains a miR172-binding site but lacks any AP2 domains. Translation of the sequence yields an amino acid sequence weakly similar to the *Arabidopsis* AP2-like protein TARGET OF EAT2 (TOE2). The presence of the miR172-binding site and the similarity to *TOE2* suggest that this cDNA corresponds to an *AP2*-like gene in *N. benthamiana*. Therefore, we name this gene *NbAP2-like1*.

Protein extraction and Western blot analysis

Ground floral tissue, saved from RNA isolations, was boiled for 5 min in 2 volumes of 2× SDS-PAGE sample buffer and centrifuged for one minute to pellet plant debris. Protein

samples were resolved by SDS-PAGE on 10% polyacrylamide gels and blotted onto nitrocellulose membrane (Biorad). The blots were briefly stained with Ponceau S and the predominant ribulose bisphosphate carboxylase (RUBISCO) bands were used as a loading control. The blots were blocked with 5% nonfat milk, probed with rabbit polyclonal antibodies against *Arabidopsis* AP2 and Goat Anti-rabbit Horseradish Peroxidase (GAR-HRP) conjugate and treated with the chemiluminescence reagents of the ECL Plus kit (Amersham).

To generate polyclonal antibodies against *Arabidopsis* AP2, the part of the cDNA encoding an N-terminal region of AP2, which is divergent among *AP2* homologs, was amplified by PCR from *AP2* cDNA and cloned into the pET-28a (Novagen) vector to incorporate an N-terminal His tag. The primers used were: AP2p31-1y, gga att cCA TAT GTG GGA TCT AAA CG ACG CAC CAC AC and AP2p31-2y, ccg ctc gag GCT CCA CTA CGG CAG CGG CAA C. Induction of expression was carried out at 30°C for 3 h in the *E. coli* strain BL21(DE3) Codon Plus. The expressed protein was purified in the native form with a fusion protein purification kit (Amersham). The rabbit anti-AP2 antisera were generated against the purified protein at Cocalico Biological, Inc.

Autoradiography and plant photography

Nucleic acid and protein blots were exposed to Kodak Biomax film for autoradiography. Radiolabeled bands were quantified using ImageQuant software. Plant images were acquired using a FUJI digital camera HC-300Z.

Results

Generation of *N. benthamiana* lines transformed with *Arabidopsis AP2* and *MIR172* genes

To study the activity of the *AP2*/miR172 regulatory circuit in *N. benthamiana*, transgenic *N. benthamiana* lines expressing *Arabidopsis AP2* and miR172 under the control of the double 35S promoter were generated (Table 1). These included twenty eight *35S::AP2* lines transformed with wild type *AP2* cDNA; twenty two *35S::AP2m3* lines transformed with mutant *AP2* cDNA containing 6 mismatches to miR172; and twenty nine *35S::MIR172* lines transformed with the *MIR172a-1* gene. Twenty-five lines transformed with the empty vector pMAT137hm were generated as controls. The presence of transgenes was confirmed by DNA gel blot analysis using a probe specific for the 35S promoter. For each construct, twenty-two plants were analyzed and all hybridized with the 35S promoter probe thus confirming stable transformation (data not shown). DNA from a wild type plant, included as a negative control, did not give any hybridization signal. The lines used in subsequent analyses were primary transformants (T0) except for flowering time and *NbDEF* and *NbGLO* RT-PCR analyses, where T1 progeny were used.

Levels of transgenic *AP2* mRNA and protein were low in *35S::AP2* lines and high in *35S::AP2m3* lines

The expression of *AP2* in *N. benthamiana* transgenic lines was analyzed by RNA and protein gel blotting using *Arabidopsis* full length cDNA probe and anti-AP2 polyclonal antibodies, respectively (Fig. 1). The *AP2* probe only detected transgenic *AP2* mRNA in *35S::AP2* and *35S::AP2m3* lines and gave no signal in *35S::MIR172* or vector lines (Fig. 1A), indicating that the *Arabidopsis AP2* probe does not hybridize to *N. benthamiana AP2* homologue(s) under our experimental conditions. Overall, *AP2* mRNA and protein accumulated to lower levels in *35S::AP2* lines compared to those in *35S::AP2m3* lines (Fig. 1). The levels of *AP2* mRNA (Fig. 1A) largely correlated with levels of AP2 protein (Fig. 1B). For instance, while AP2 protein was not detectable in *35S::AP2* lines that had low

levels of *AP2* mRNA (lines 2, 3, 5, 6, 9, 20 & 21), the few *35S::AP2* lines that had higher levels of *AP2* mRNA (lines 1, 4 & 7) accumulated detectable levels of protein (Fig. 1).

Accumulation of transgenic and endogenous miR172 in *N. benthamiana*

The accumulation of miR172 in *N. benthamiana* transgenic lines was analyzed by miRNA gel blot hybridization using *Arabidopsis* miR172 probe (Fig. 2). Interestingly, miR172 was detectable not only in *35S::MIR172* lines transformed with *Arabidopsis* miR172 but also in *35S::AP2*, *35S::AP2m3* and vector lines (Fig. 2). This indicates that the *N. benthamiana* endogenous miR172 (*Nb*miR172) is detectable using the *Arabidopsis* miR172 probe, thus revealing conservation of the *MIR172* gene, and possibly *AP2*-like endogenous targets, in *N. benthamiana*. The levels of endogenous miR172 were comparable in *35S::AP2*, *35S::AP2m3* and vector lines while most characterized *35S::MIR172* lines over-expressed miR172 up to an average of 7- and 14-fold compared to vector lines at 15 days and 60 days post-flowering, respectively (Fig. 2B). *35S::MIR172* lines 2, 3, 4 and 9 were moderately over-expressing compared to the highly over-expressing lines 6, 7 & 11 (Fig. 2A). Lines 1, 5 & 8 accumulated miR172 at levels comparable to vector lines (Fig. 2A). None of the over-expressing lines showed developmental silencing of the miR172 transgene within the duration of plant growth (Fig. 2B).

Arabidopsis AP2 induces floral patterning defects in *N. benthamiana*

Expression of miR172-resistant *Arabidopsis AP2* in *N. benthamiana* (*35S::AP2m3* lines) induced floral patterning defects and the severity of the defects correlated with *AP2* mRNA and protein levels (Figs. 3 and 4). The phenotypes were characterized by reduced petal size and short-and-bulged carpels in at least 60% of the flowers of most *35S::AP2m3* lines (Fig. 3, panels C, D, F & I) and reduced fertility. Some *35S::AP2m3* lines with highest levels of AP2 protein such as lines 1, 4, 6, 14 & 16 (Fig. 1B) were severely phenotypic, producing flowers with numerous stamens and carpels (Fig. 4, panels G & H), followed by emergence of numerous petals in all flowers in late stages of development (Fig. 4, panel I). All the flowers produced by these lines were severely phenotypic and completely sterile. These phenotypes were reminiscent of *Arabidopsis* lines expressing *35S::AP2m3*. On the other hand, most *N. benthamiana* lines transformed with miR172-susceptible *AP2* (*35S::AP2* lines) did not accumulate *AP2* mRNA or protein (Fig. 1) and were essentially non phenotypic and completely fertile. The only exceptions included lines 1, 4 & 7, which moderately accumulated *AP2* mRNA and protein and had a mild phenotype characterized by reduced petal size in less than 5% of the flowers of each plant (Fig. 3, panels B & G). Surprisingly, *35S::AP2* line 7 shows similar *AP2* mRNA and protein levels as *35S::AP2m3* lines 8, 12 & 14 but its phenotype is weaker. Overall, the expression levels of wild type and mutant *AP2* transgenes together with the general absence of phenotypes in *35S::AP2* plants and the high frequency and severity of phenotypes in *35S::AP2m3* plants are consistent with a regulatory activity of an uncharacterized endogenous miR172 encoded by *N. benthamiana*.

The proliferation of reproductive organs in *35S::AP2m3* lines is reminiscent of loss of floral determinacy previously seen in *Arabidopsis ag* mutants (Bowman et al. 1991). In addition to specifying stamen and carpel identities, *Arabidopsis AG* controls floral determinacy by repressing the expression of *WUS*, a gene that maintains stem cell fates (Laux et al. 1996; Schoof et al. 2000; Lenhard et al. 2001; Lohmann et al. 2001; Baurle and Laux 2005; Kieffer et al. 2006). To determine if the loss of floral determinacy in *35S::AP2m3* lines could be due to reduced expression of *NbAG*, we identified *NbAG* and studied its mRNA levels by semi-quantitative RT-PCR (Fig. 4J). The levels of *NbAG* mRNA (Fig. 4J, left panel) in *35S::AP2m3* plants were comparable to those in vector plants or *35S::AP2* plants. With a similar approach, we found the levels of *NbWUS* to be comparable among vector alone, *35S::AP2*, and *35S::AP2m3* plants (Fig. 4J, right panel).

Floral homeotic transformations induced by *Arabidopsis* miR172 in *N. benthamiana*

Ectopic expression of *Arabidopsis* miR172 induced novel floral patterning defects including sepal-to-petal transformations in *N. benthamiana* (Fig. 5). The phenotypes correlated with miR172 expression levels. *35S::MIR172* lines with miR172 levels comparable to endogenous miR172 levels in vector controls (Fig. 2, *35S::MIR172* lines 1, 5 & 8) were non phenotypic (not shown). Moderately over-expressing lines such as *35S::MIR172* lines 2, 3, 4, & 9 (Fig. 2) produced a subset of flowers with increased number of sepals and petals (Fig. 5A), with such flowers appearing late and at a low frequency (~15% per plant). Highly over-expressing miR172 lines 6, 7 & 11 produced a subset of flowers (~50%) with a range of partial-to-complete sepal-to-petal transformations (Fig. 5B and C) and ripples at the base of the first whorl organs (Fig. 5B: inset). The phenotypic flowers were observed at the onset of flowering and throughout the progression of plant growth. None of the miR172 phenotypes shown in Fig. 5 were observed in vector control, *35S::AP2* or *35S::AP2m3* lines. The sepal-to-petal transformation was not found in *Arabidopsis* plants over-expressing *MIR172* genes (Aukerman and Sakai 2003; Chen 2004). Transgenic miR172 levels remained high through the entire duration of plant growth (Fig. 2B), thus excluding the possibility of the appearance of phenotypic flowers being due to the onset of transgene silencing at the miR172 locus in late stages of development.

Overall, the first whorl transformations in *35S::MIR172* plants are reminiscent of ectopic B function in the first whorl. In an attempt to dissect the molecular basis of the *35S::MIR172* phenotypes, we studied the expression patterns of *N. benthamiana* B function genes *NbDEF* and *NbGLO*, which are orthologous to A*rabidopsis* B function genes *APETALA3 (AP3)* and *PISTILLATA (PI)*, respectively (Liu et al. 2004). Ectopic expression of B function genes, *PI* in *Arabidopsis* (Krizek and Meyerowitz 1996) and *GREEN PETAL* in *Petunia* (Halfter et al. 1994), induced sepal-to-petal transformations. We found that the levels of both *NbDEF* and *NbGLO* mRNA were significantly enhanced in sepals of *35S::MIR172* plants compared to vector control plants (Fig. 6A). Note that the sepals examined were dissected off young floral buds before any visual indication of homeotic transformation.

It is conceivable that the *Arabidopsis* miR172 may cause the floral defects through its regulation of *NbAP2-like* targets. We were able to identify an *AP2*-like gene from *N. benthamiana, NbAP2-like1*, which contains a miR172 target site. The comparison of the miR172 target sites in *Arabidopsis AP2* and *AP2-like* genes of *N. benthamiana* and related *Solanaceae* species, *P. hybrida* and *L. esculentum*, is shown in Fig. 6B. The 21-nucleotide target site of miR172 in *NbAP2-like1* has a C → T substitution at position 20 that introduces a mismatch to *Arabidopsis* miR172 at the second nucleotide from the 5′ end of miR172 (Fig. 6B). To determine if transgenic *Arabidopsis* miR172 had any effect on *NbAP2-like1* levels, we examined *NbAP2-like1* mRNA by Northern blot hybridization of RNA isolated from sepals of *35S::MIR172* and vector plants (Fig. 6C). Using a probe designed to span the miR172 binding site, a ~2 kb mRNA was detected along with ~1.2 and ~0.6 kb fragments, which might be putative 5′ and 3′ miR172-mediated cleavage products (Fig. 6C). The levels of *NbAP2-like1* transcripts in *35S::MIR172* plants were similar to vector plants indicating that over-expression of *Arabidopsis* miR172 had no significant effect on *NbAP2-like1* mRNA levels.

Arabidopsis 35S::MIR172 plants were found to be early flowering (Aukerman and Sakai 2003; Chen 2004). The effect of miR172 over-expression on flowering time in *N. benthamiana* was not apparent in primary transformants and was studied in thirty T1 progeny of *35S::MIR172* line # 7 and thirty T1 progeny of a vector line grown at 25°C under continuous light. All the *35S::MIR172* plants flowered after 14 days following transfer from kanamycin plates to soil compared to 24 days for vector plants. Thus miR172 over-expression caused early flowering not only in *Arabidopsis* but also in *N. benthamiana*.

Discussion

Mode of regulation of *AP2* by miR172

The expression of miR172-resistant *Arabidopsis AP2* in *N. benthamiana* resulted in elevated *AP2* mRNA and protein levels and severe floral patterning defects. On the other hand, *N. benthamiana* plants transformed with the wild type *AP2* cDNA hardly accumulated any *AP2* mRNA or protein and were essentially non phenotypic like vector control plants. Thus the observed *35S::AP2m3* phenotypes could be specifically attributed to the expressed mutant *AP2* transgenes. Overall, the data suggest that the expression of the *35S::AP2* transgene is controlled by an endogenous *N. benthamiana* miR172 at the level of transcript cleavage. This is in contrast with previous observations in *Arabidopsis* in which the endogenous and transgenic *AP2* expression was shown to be translationally repressed (Aukerman and Sakai 2003; Chen 2004; Schwab et al. 2005).

These observations do not necessarily indicate a difference in the mode of miR172-mediated regulation of *AP2*. It should be noted that miR172-guided cleavage of *AP2* mRNA also occurs in *Arabidopsis*, although this mode of regulation is insufficient to result in the down regulation of *AP2* expression, presumably due to feedback transcriptional regulation (Aukerman and Sakai 2003; Schwab et al. 2005). Translational inhibition by miR172 ensures reduced levels of AP2 protein despite limited changes in *AP2* mRNA levels. We envision two possibilities to explain the apparent difference in mode of regulation when *35S::AP2* was expressed in *Arabidopsis* and in *N. benthamiana*. First, *N. benthamiana* miR172, which may be slightly different in sequence from *Arabidopsis* miR172, may be more efficient in guiding the cleavage of *Arabidopsis AP2* mRNA. Second, the observed difference in transgenic *AP2* in the two species may not reflect a difference in the mode of regulation of the endogenous miR172/*AP2* pair in the two species. Rather, the reduced mRNA levels of *35S::AP2* in *N. benthamiana* may be largely due to secondary RNA silencing triggered by the initial *Nb*miR172-guided cleavage of the transgenic *Arabidopsis AP2* mRNA. It is known that transgenes containing miRNA target sites tend to undergo post-transcriptional gene silencing (Parizotto et al. 2004). Interestingly, over-expression of *Arabidopsis* miR172 in *N. benthanimana* does not lead to a reduction in the levels of the endogenous *NbAP2-like1* mRNA levels. Perhaps, *Arabidopsis* miR172 also leads to translational regulation of *NbAP2-like1*.

Loss of floral determinacy in *N. benthamiana* transformed with *35S::AP2m3*

The proliferation of numerous petals, stamens and carpels in severely phenotypic *35S::AP2m3* plants represents a recapitulation of the effect of the same transgene in *Arabidopsis* as described previously (Chen 2004). Although the phenotypes resemble *Arabidopsis ag* mutants in loss of floral determinacy (Bowman et al. 1991), the expression of *NbAG* was not altered at the RNA level in *N. benthamiana 35S::AP2* and *35S::AP2m3* plants compared to vector plants. Similarly, the levels of *Arabidopsis* AG protein were not altered in *Arabidopsis 35S::AP2* and *35S::AP2m3* plants compared to vector plants (Zhao and Chen, unpublished data). This indicates that *35S::AP2m3* leads to floral stem cell defects independently of *AG*. Consistent with this, a recent study uncovered a role for *Arabidopsis AP2* in meristem stem cell maintenance that is independent of the *AG* pathway (Wurschum et al. 2006). Although we did not detect any difference by RT-PCR in *NbWUS* RNA levels among *35S::AP2m3*, *35S::AP2*, and vector plants, we cannot exclude a role of *WUS* in mediating the stem cell defects in *35S::AP2m3*. Since *WUS* is expressed in only a small number of meristematic cells, the large increase in floral organ number in *35S::AP2m3* would dilute the proportion of *WUS*-expressing cells.

Floral homeotic transformations and early flowering in *N. benthamiana* expressing *Arabidopsis* miR172

Our studies not only revealed the presence of *N. benthamiana*-encoded miR172 but also suggested close similarity between the *Arabidopsis* and *N. benthamiana MIR172* genes and hence target sites in *AP2* homologues. However, the gene(s) that encode *N. benthamiana* miR172 and their *AP2*-like targets have yet to be isolated.

In *Arabidopsis* two *AP2*-like targets of miR172, *TARGET OF EAT1* (*TOE1*) and *TOE2*, function in flowering time control as floral repressors, and ectopic expression of miR172 causes early flowering likely due to repression of *TOE1* and *TOE2* and other redundant *AP2*-like targets of miR172 (Aukerman and Sakai 2003). The early flowering phenotype of *Arabidopsis 35S::MIR172* plants was recapitulated in *35S::MIR172 N. benthamiana* lines suggesting that *N. benthamiana* homologues of *TOE* genes are regulated by miR172 and serve as floral repressors.

We predicted that over expression of *Arabidopsis* miR172 in *N. benthamiana* would repress an *N. benthamiana AP2* ortholog, *NbAP2*, to result in phenotypes reminiscent of *Arabidopsis ap2* mutants (Bowman et al. 1991). However, the phenotypes of *35S::MIR172* lines, i.e., the sepal-to-petal transformation and increase in sepal and petal number, are different from those of *Arabidopsis ap2* mutants. *35S::MIR172* plants accumulated higher levels of *NbDEF* and *NbGLO* transcripts in sepals compared to vector controls prior to visible homeotic transformation. This indicates that sepal-to-petal transformations are caused by ectopic B function in the first whorl. The phenotypes could not be attributed to co-suppression at the miR172 locus (which could potentially lead to increased *NbAP2* activity) since miR172 was highly expressed in phenotypic flowers and throughout the duration of plant growth.

It is not clear how transgenic miR172 increases the expression of B function genes in the first whorl. We consider the following three hypotheses. First, over-expression of *Arabidopsis* miR172 leads to reduced *NbAP2* expression to result in ectopic B function in the first whorl. In this scenario, *NbAP2* normally serves as a cadastral gene that represses the activity of B function in the first whorl and that *NbAP2* is not necessary for perianth identity. Second, over-expression of *Arabidopsis* miR172 leads to increased *NbAP2* expression to result in ectopic B function in the first whorl. From the partial sequence of *NbAP2-like1*, we identified a mismatch between miR172 target site in *NbAP2-like1* mRNA and transgenic *Arabidopsis* miR172 at the second nucleotide from the 5′ end of miR172. Perfect complimentarity at this 5′ region of miRNAs has been previously shown to be critical for miRNA function (Mallory et al. 2004b). If *NbAP2* also has such a mismatch to *Arabidopsis* miR172, it seems possible that *Arabidopsis* miR172 may not be having a repressive effect on *NbAP2* but instead could be sequestering *NbAP2* mRNA from being regulated by *NbmiR172* to result in increased *NbAP2* activity. However, increased AP2 levels in *Arabidopsis* do not lead to sepal-to-petal transformations (Chen 2004). Third, the *35S::MIR172* phenotypes could be largely due to repression of other *N. benthamiana* miR172 target genes, such as the *NbAP2-like1* that we identified and perhaps other as yet unknown *NbAP2-like* genes, by transgenic miR172. Regardless of which hypothesis is correct, the functions of *AP2* and *AP2-like* genes in floral patterning in *Arabidopsis* and *N. benthamiana* must be divergent.

AP2/miR172 transgenic system as a potential functional genomic tool

The phenotypes induced by the *Arabidopsis AP2* and miR172 transgenes in *N. benthamiana* are restricted to flower development. This is in spite of the ubiquitous 35S promoter mediated expression of *AP2*/miR172 transgenes in both vegetative and reproductive tissue.

This offers an effective means of altering plant floral architecture in different plant species without any impairment in normal plant development. Thus the technology could yield plants with improved ornamental traits such as increased petal number and reduced pollen production and hence diminished allergenicity. In addition, the induction of early flowering by miR172 over-expression in different plant species could be a useful tool in agriculture and forestry.

Our results also indicate that the *Arabidopsis* wild type *AP2* transgene, and not the mutant form, is subject to regulation by an endogenous miR172 in a heterologous plant species *N. benthamiana*. The *35S::AP2/AP2m3* system thus offers a means to probe the activity of unidentified endogenous miR172 homologues and to evaluate the conservation or divergence of floral homeotic gene function in different plant species.

Acknowledgments

We are indebted to Dr Vicki Bowman Vance for support to S.M. during the revision of the manuscript. We are grateful to Drs Zora Svab and Pal Maliga for plant growth facilities; Dr Zora Svab for advice on tobacco tissue culture; Michael Petercsak for green house plant care; and Dr Hailing Jin for *N. benthamiana* cDNAs. We thank Drs Hailing Jin, Li Zhao, Bin Yu and Julien Curaba for helpful discussions, and Drs Vicki Bowman Vance and Beth Krizek for critical reading of the manuscript. S.M. was supported by a postdoctoral fellowship from the Waksman Institute at Rutgers University. The research was supported by a NIH grant (GM61146) to X.C.

Abbreviations

AG	AGAMOUS
AP2	APETALA2
CaMV	cauliflower mosaic virus
DEF	DEFICIENS
GLO	GLOBOSA
HMW RNA	high molecular weight RNA
LMW RNA	low molecular weight RNA
miRNA	microRNA
miR172	microRNA172
NAG1	Nicotiana tabacum AGAMOUS
NbAG	Nicotiana benthamiana AGAMOUS
NbAP2	Nicotiana benthamiana APETALA2
NbDEF	Nicotiana benthamiana DEFICIENS
NbGLO	Nicotiana benthamiana GLOBOSA
NbWUS	Nicotiana benthamiana WUSCHEL
RUBISCO	ribulose bisphosphate carboxylase
TOE	TARGET OF EAT
WUS	WUSCHEL

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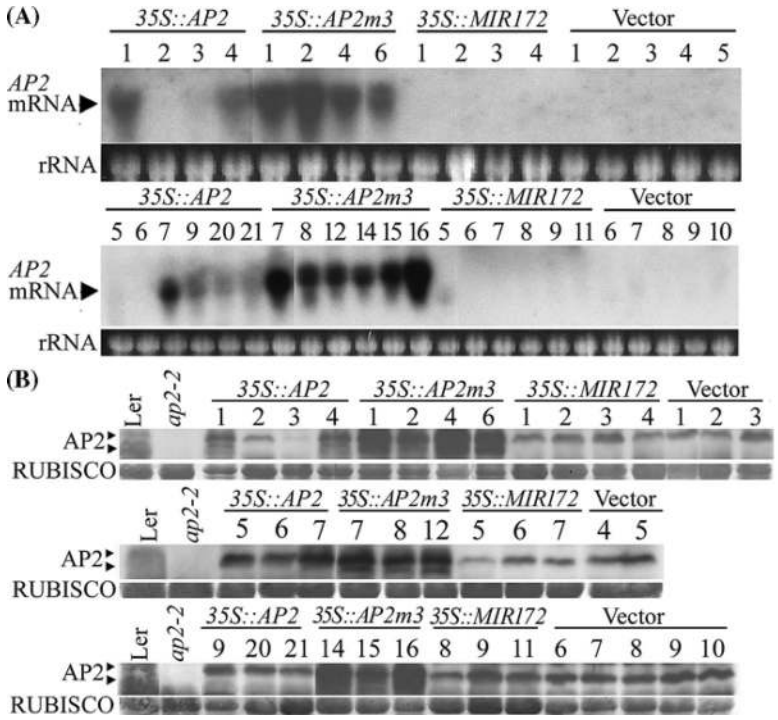

Fig. 1.

Analysis of *AP2* transgene expression in *35S::AP2*, *35S::AP2m3*, *35S::MIR172* & Vector transgenic lines. (**A**) RNA gel blot analysis of *AP2* mRNA levels. Total RNA was isolated from floral buds of transgenic lines (indicated above each blot) 15 days post flowering and separated into high and low molecular weight fractions. 10 μg of HMW RNA was used in Northern blotting with P^{32}-labelled full length *Arabidopsis AP2* cDNA as the probe. Ethidium bromide stained rRNA is shown as loading control for each blot. (**B**) Protein gel blot analysis of AP2 protein levels in transgenic lines analyzed in Fig. 1A. Total protein was isolated from part of the ground floral bud tissue used to isolate RNA that was used in Fig. 1A and probed with *Arabidopsis* AP2 polyclonal antibodies. Wild-type *Arabidopsis* (Landsberg *erecta*–L*er*) was included as a positive control showing two closely migrating AP2 forms that are absent in the *Arabidopsis ap2-2* mutant. The anti-AP2 antibody recognized an unknown protein from *N. benthamiana* that migrated together with the slower migrating form of *Arabidopsis* AP2 (upper arrow), since the band was present in vector alone lines. In most *35S::AP2m3* lines, both bands increased in intensity indicating that both *Arabidopsis* AP2 protein forms accumulated to higher levels as compared to vector lines. In the second and third panels, the two bands in L*er* were slightly offset in mobility from the rest of the bands due to the "smiling" of the gels. Ponceau S-stained RUBISCO is shown as a loading control for each blot

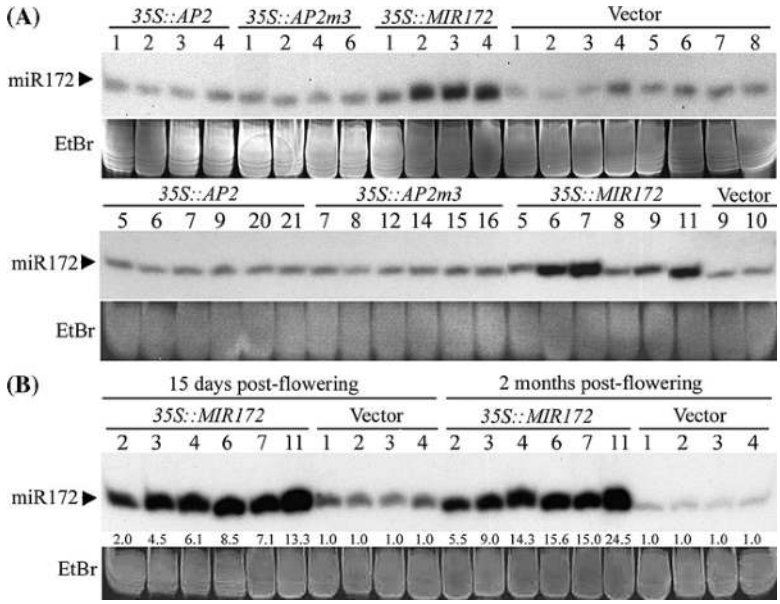

Fig. 2.

RNA gel blot analysis of miR172 levels in *35S::AP2*, *35S::AP2m3*, *35S::MIR172* & Vector transgenic lines analyzed in Fig. 1. (**A**) Levels of miR172 15 days post flowering. The predominant slow migrating RNA species, stained with ethidium bromide prior to blotting, is shown as loading control. (**B**) Levels of miR172 in early compared to very late flowers of *35S::MIR172* lines. The relative levels of miR172 are indicated for each sample, with levels in vector control samples designated as 1.0

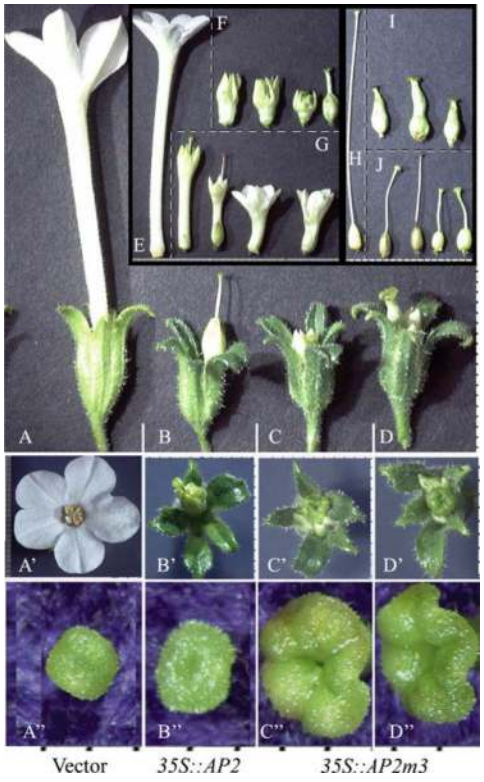

Fig. 3.

Phenotypes of *35S::AP2* & *35S::AP2m3* lines. Whole flower side view (**A**) and top view (**A**″), and stigma (**A**″) of vector line. Whole flower side view (**B**) and top view (**B**′), and stigma (B″) of *35S::AP2* flowers that have a mild phenotype associated with moderate accumulation of *AP2* mRNA and protein [e.g. *35S::AP2* lines 1, 4, & 7 (Fig. 1)]. Whole flower side view (**C & D**) and top view (C′ & D′), and stigma (C″ & D″) of *35S::AP2m3* flowers that have a strong phenotype associated with high accumulation of *AP2* mRNA and protein (observed for nearly all *35S::AP2m3* lines analyzed in Fig. 1). Sepals were removed to reveal reduction in petal size of phenotypic *35S::AP2* (**G**) and *35S::AP2m3* (**F**) lines as compared with the wild type phenotype of vector lines (**E**). Petals were further removed to reveal enlarged carpels of strongly phenotypic *35S::AP2m3* lines (**I**) as compared to midly affected carpels of *35S::AP2* (**J**) and non-affected carpels of vector lines (**H**). All flowers were photographed 15 days post flowering. Each unit on the ruler is equivalent to 1 mm for all images (Fig. 1 through Fig. 5)

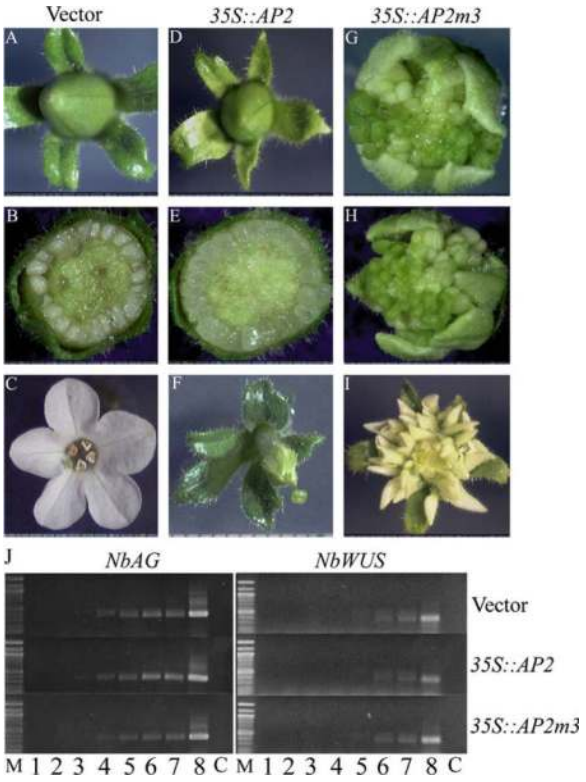

Fig. 4.

Loss of floral determinacy in severely phenotypic *35S::AP2m3* lines. Vector: Top view (**A**) and section (**B**) of maturing ovary, and top view (**C**) of very late flower. *35S::AP2*: Top view (**D**) and section (**E**) of maturing ovary, and top view (**F**) of very late flower. **D–F** are from mildly phenotypic *35S::AP2* lines described in Fig. 3. *35S::AP2m3*: A subset of phenotypic *35S::AP2m3* lines with highest levels of AP2 protein (lines 1, 4, 6, 14, & 16 in Fig. 1B) were severely phenotypic, producing flowers with numerous stamens and carpelloid structures (**G** & **H**) and numerous petals (**I**, very late flower). Very late flowers (C, F & I) were collected from plants for photography 2 months post flowering. The rest were collected 15 days post flowering. (**J**) Semi-quantitative RT-PCR analyses of levels of *NbAG* (left panel) and *NbWUS* (right panel) in vector, *35S::AP2* & *35S::AP2m3* lines. For both panels, lanes 1–8 correspond to samples from PCR cycles 12, 15, 18, 21, 24, 27, 30 and 40, respectively. Lane C (control): reverse transcriptase-lacking cDNA synthesis reaction used as PCR template. Lane M (marker): 100 bp DNA ladder

Fig. 5.

Phenotypes of *35S::MIR172* lines. (**A**) Vector line as compared with *35S::MIR172* flowers with increased number of sepals and petals typical of a subset of flowers of moderately over-expressing *35S::MIR172* lines 2, 3, 4 & 9 shown in Fig. 2. (**B**) Vector line as compared with phenotypic *35S::MIR172* flower with sepal-to-petal transformations and ripples at the base of the first whorl perianth (inset) typical of highly over-expressing *35S::MIR172* lines 6, 7 & 11 shown in Fig. 2. (**C**) Vector line as compared with *35S::MIR172* flowers that show a range of partial to complete sepal-to-petal transformations associated with highly over-expressing *35S::MIR172* lines 6, 7 & 11. In addition to sepalto-petal transformations, these lines also have an increased number of sepals and petals for a subset of flowers (not shown)

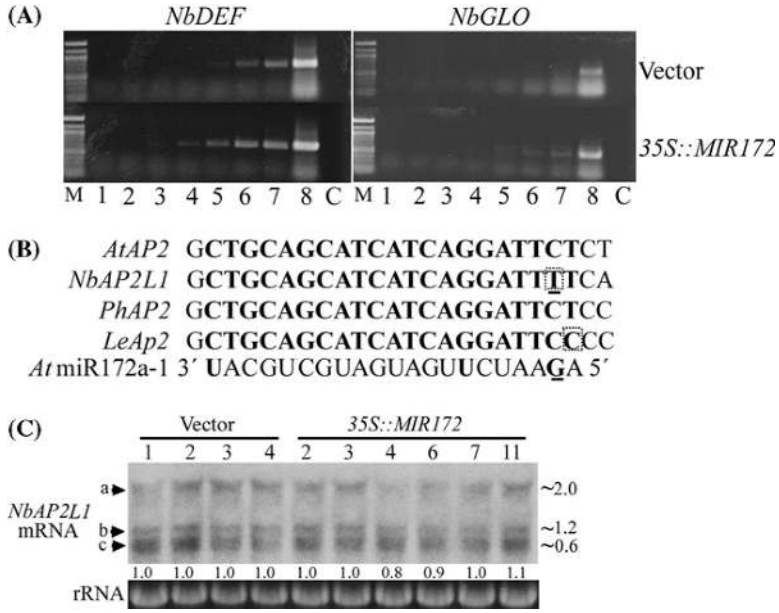

Fig. 6.

Expression analysis of **NbAP2-like1** and B function genes **NbGLO** and **NbDEF**. (**A**) Semi-quantitative RT-PCR analysis of levels of *NbDEF* (left panel) and *NbGLO* (right panel) in young sepals of vector and *35S::MIR172* line # 7. Lanes 1–8, C & M are as described in Fig. 4J. (**B**) Alignment of the *NbAP2-like1* (*NbAP2L1*) target site with *Arabidopsis AP2* (*AtAP2*) and *AP2* cDNAs of *Solanaceae* species *P. hybrida* (*PhAP2*) and *L. esculentum* (*LeAP2*). The 21-nucleotide core target site is indicated in bold, with non-conserved nucleotides within the 21-nucleotide core target site boxed. The sequence *of Arabidopsis* miR172 (*At*miR172a-1) is shown below the alignment, with 3 mismatched nucleotides shown in bold and the mismatch that is unique only to the *At*miR172/*NbAP2L1* pair underlined. (**C**) RNA gel blot analysis of *NbAP2-like1* in vector & *35S::MIR172* lines. Total RNA was isolated from newly formed sepals of vector & *35S::MIR172* T1 lines (indicated above the blot). 10 μg of HMW RNA was resolved on denaturing gels, blotted onto membranes (Amersham) and probed with ^{32}P-labelled *NbAP2L1* antisense RNA probe. a, full length *NbAP2L1* mRNA (~2.0 kb); b, putative 5′ cleavage fragment (~1.2 kb); c, putative 3′ cleavage fragment (~0.6 kb). Ethidium bromide stained rRNA is shown as loading control. The relative levels of the ~2 kb full-length *NbAP2L1* mRNA are indicated for each sample (levels in vector control samples are designated as 1.0)

Table 1

Independent *N. benthamiana* lines transformed with *Arabidopsis AP2* and *MIR172* genes

Transgenic line	Strong phenotype	Mild phenotype	Wild type phenotype	Total
35S::AP2	0	6[b]	22	28
35S::AP2m3	12[a]	4[b]	6	22
35S::MIR172	4[c]	11[a]	14	29
Vector	0	0	25	25

[a]Number of lines with enlarged carpels and/or numerous petals, stamens and carpels

[b]Number of lines with reduced petal size in less than 5% of flowers of each plant

[c]Number of lines with sepal-to-petal transformations and increased number of sepals and petals

[d]Number of lines with increased number of sepals and petals only