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LEUNIG and SEUSS co-repressors regulate *miR172* expression in *Arabidopsis* flowers

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SUMMARY

Central to the ABCE model of flower development is the antagonistic interaction between class A and class C genes. The molecular mechanisms underlying the A-C antagonism are not completely understood. In *Arabidopsis thaliana*, miR172 is expressed in the inner floral whorls where it downregulates the class A gene *APETALA 2* (*AP2*). However, what controls this predominantly inner whorl-specific expression of miR172 is not known. We show that the LEUNIG (LUG) and SEUSS (SEU) co-repressors repress *miR172* expression in the outer whorls of *A. thaliana* flowers. The recruitment of LUG/SEU to the *miR172* promoters is dependent on AP2, suggesting that AP2 represses the expression of its cognate microRNA. Our study provides new insights into the molecular mechanisms underlying the A-C antagonism and shed light on the transcriptional regulation of *miR172* during flower development.

KEY WORDS: APETALA 2, Co-repressors, Flower development, LEUNIG, SEUSS, miR172, *Arabidopsis*

INTRODUCTION

The ABCE model of flower development (Coen and Meyerowitz, 1991; Krizek and Fletcher, 2005; Theissen and Saedler, 2001) successfully explains how floral organ identity is established through the combinatorial action of A, B, C and E classes of genes, all of which, except *APETALA 2* (*AP2*), encode MADS box transcription factors. Central to the model is the antagonistic interaction between the A and C class genes. Whereas class A activity is repressed by class C genes in the inner two whorls, class C activity is repressed by class A genes in the outer two whorls (Bowman et al., 1991; Coen and Meyerowitz, 1991; Drews et al., 1991).

The class C gene *AGAMOUS* (*AG*) encodes a transcriptional repressor of the class A gene *APETALA 1* (*AP1*) in the inner two whorls (Gustafson-Brown et al., 1994; Mandel et al., 1992). Two interacting transcriptional co-repressors, LEUNIG (LUG) and SEUSS (SEU), have been shown to repress *AG* transcription in the outer two whorls (Conner and Liu, 2000; Franks et al., 2002; Liu and Meyerowitz, 1995; Sridhar et al., 2004). Neither LUG nor SEU contains any known DNA-binding domains; their binding to the *AG* cis-regulatory elements is dependent on a direct physical interaction between SEU and the two MADS box proteins AP1 and SEPALLATA 3 (SEP3) (Gregis et al., 2006; Gregis et al., 2009; Sridhar et al., 2006).

AP2 is another transcriptional repressor of *AG* (Bowman et al., 1991; Drews et al., 1991). Using chromatin immunoprecipitation, AP2 was shown to directly bind within the *AG* second intron (Yant et al., 2010). Ectopic AP2 activity via the *pAP3:AP2r* transgene resulted in the repression of *AG* mRNA, supporting a direct repression of *AG* by AP2 (Wollmann et al., 2010). It is not yet known whether the repressive activity of AP2 depends on its recruitment of transcriptional co-repressors such as LUG/SEU.

The outer whorl-specific activity of *AP2* has been largely attributed to a microRNA (miRNA), miR172, that specifically blocks *AP2* mRNA translation as well as causing *AP2* mRNA cleavage in the inner two whorls (Aukerman and Sakai, 2003; Chen, 2004; Schwab et al., 2005). In situ hybridization has revealed that miR172 is expressed most strongly in the inner whorls (Chen, 2004). A recent re-examination by in situ hybridization revealed that the spatial expression patterns of *AP2* mRNA and miR172 are largely complementary, although there is a transient overlap in the second and possibly third floral whorls (Wollmann et al., 2010). Nevertheless, what determines the inner whorl-specific expression of miR172 remains unknown. A genome-wide analysis of AP2 binding sites using ChIP-seq indicated that *miR172b* is a target of AP2 (Yant et al., 2010), suggesting a possible role of AP2 in *miR172* regulation.

Here, we show that LUG directly and negatively regulates *miR172c* and *miR172e* expression in sepals. This direct repression of *miR172* by LUG also requires SEU and AP2, suggesting that AP2 might recruit the SEU-LUG co-repressor complex to repress *miR172* transcription in the outer floral whorls. Our study provides important insights into miRNA regulation and suggests a positive-feedback loop, in which AP2 maintains its own outer whorl-specific activity by repressing the expression of its cognate miRNA.

MATERIALS AND METHODS

Plant materials

lug-3, *seu-1* and *ap2-2* mutants and *35S:GFP-LUG; lug-16*, *ProSEU:GFP-SEU; seu-1*, *ProSEU:GFP-SEU; ap2-2* and *35S:AP2m3* transgenic lines, all in the Landsberg *erecta* (L*er*) background, were grown at 20°C under long-day conditions (16 hours light/8 hours dark). *35S:GFP-LUG* in *pAVA393* (Conner and Liu, 2000) was excised with *Hin*dIII and *Sac*I and cloned into *pCAMBIA2300* (Cambia, Brisbane, Australia), which was used to transform and rescue *lug-16*. *ProSEU:GFP-SEU*, previously shown to rescue *seu-1* (Azhakanandam et al., 2008), was crossed into *ap2-2*+/+*ant-9* to yield *ProSEU:GFP-SEU (WT)* and *ProSEU:GFP-SEU (ap2-2)*. *35S:AP2m3* was described previously (Chen, 2004; Zhao et al., 2007).

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RNA expression

RNA was isolated from inflorescences using TRI reagent (Sigma-Aldrich, St Louis, MO, USA). For small RNA blots, 30 μg total RNA was separated on a 15% acrylamide gel, transferred, and cross-linked onto Hybond-N nylon membrane using EDC [N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride; Sigma-Aldrich] (Pall and Hamilton, 2008). [γ^{32}P]ATP and the mirVana Probe & Marker Kit (Ambion, Austin, TX, USA) were used to label the *miR172* oligo (see Table S1 in the supplementary material). The small RNA blot was hybridized and washed as previously described (Lau et al., 2001; Lee and Ambros, 2001).

For northern blots, PCR-amplified *AP2* first exon or 5S RNA was labeled with [α^{32}P]dCTP (Perkin-Elmer, Waltham, MA, USA) with Ready-To-Go DNA Labeling Beads (Amersham, Piscataway, NJ, USA). Then 15 μg total RNA was separated on a 1% agarose gel, transferred onto BrightStar-Plus membrane (Ambion), hybridized and washed using the Northern Max-Gly Kit (Ambion). The signal intensity was measured using Image J (NIH).

For *AP2* quantitative (q) RT-PCR (see Table S1 in the supplementary material), the QuantiSure First-Strand cDNA Kit (Accugen Bioscience, Rockville, MD, USA) was used, followed by qRT-PCR with SsoFast EvaGreen Supermix on a CFX96 real-time PCR machine (BioRad, Hercules, CA, USA). The Ct^{AP2} was subtracted from the Ct^{GAPC} to yield the ΔCt^{WT} and ΔCt^{lug3}. The Pfaffl formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001), where $-\Delta\Delta Ct=\Delta Ct^{WT}-\Delta Ct^{lug3}$, gave the expression difference. Error bars represent the s.d. of two biological replicates (three technical repeats each).

For Fig. S1 in the supplementary material, RNAs isolated from wild-type (L*er*) and *lug-3* inflorescence tissues were enriched for small RNAs, labeled with Cy5, and hybridized to a custom-designed miRNA chip as described (Pang et al., 2009). Average expression values were first normalized against internal positive controls (tRNAs) before they were compared between wild type and *lug-3*.

miR172 in situ hybridization

A direct tandem oligonucleotide concatamer (4×) of the sense *miR172* strand sequence 5′-AGAATCTTGATGATGCTGCAG-3′, with a T7 RNA polymerase-binding site (5′-CCCTATAGTGAGTCGTATTA-3′) at the 3′ end, served as the template for T7 transcription with the digoxygenin (DIG) RNA Labeling Mix (Roche, Basel, Switzerland). The *miR172* sense probe was made by a similar strategy. In situ hybridization was carried out based on Carr and Irish (Carr and Irish, 1997); slides were hybridized at 42°C overnight and were washed three times at 42°C for 30 minutes with 0.2× SSC.

Chromatin immunoprecipitation (ChIP)

Two to three grams of inflorescences with stage 1-11 flowers were used in ChIP based on Sridhar et al. (Sridhar et al., 2006). Protein A Dynabeads (Dynal/Invitrogen, Carlsbad, CA, USA) and a polyclonal anti-GFP antibody (AB290-50, Abcam, Cambridge, MA, USA) were used. qPCR with the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA, USA) was performed on the CFX96 qPCR machine. The percent efficiency for each primer is shown in Table S4 in the supplementary material.

Based on Mukhopadhyay et al. (Mukhopadhyay et al., 2008), the Ct value for the plus antibody sample (+AB) and for –AB was independently subtracted from the Ct value of the input to yield ΔCT. Then ΔCT^{+AB} was subtracted from ΔCT^{-AB} to yield the ΔΔCT for each sample. The Pfaffl formula was used to calculate the fold enrichment.

Yeast two- and three-hybrid assays

The Matchmaker System (Clontech, Mountain View, CA, USA) was used. *ANT-pGAD424* (Krizek and Sulli, 2006) and *AP2delta-pGAD424* were gifts of B. A. Krizek. *SEU (1-563)-pGBT9* and *LUH-pGAD424* were reported previously (Sridhar et al., 2004; Sitaraman et al., 2008). To make *AP2-pGADT7*, *AP2* was excised from *pGG30* with *Nco*I and *Eco*RI and cloned into *pGADT7*. For *P426-GAPD*, the *GAPD* promoter was excised with *Bam*HI and *Eco*RI, cloned into *pCR2.1-TOPO*, excised again with *Sac*I and *Eco*RI and cloned into *p426 GAL*, replacing *GAL*. For *P426-SEU*, *SEU* was excised from *pCRII-TOPO-SEU* (Franks et al., 2002) with *Hin*dIII and *Xho*I and cloned into *p426 GAPD* at *Hin*dIII-*Sal*I. Constructs were introduced one at a time into *Saccharomyces cerevisiae* strain PJ694A according to the Yeast Protocols Handbook (Clontech).

Bimolecular fluorescence complementation (BiFC)

AP2, *SEU* and *LUH* were cloned into *pUC-SPYNE* and *pUC-SPYCE* (Walter et al., 2004). Specifically, full-length *AP2*, *SEU* or *LUH* was amplified (for primers, see Table S1 in the supplementary material), cloned into *pGEM-T Easy* (Promega, Fitchburg, WI, USA) for *AP2* and *SEU* or into *pCR8/GW/TOPO* (Invitrogen) for *LUH*, excised with *Sal*I and *Xma*I, and then cloned into *pUC-SPYNE* and *pUC-SPYCE*. Helios Gene Gun (BioRad) was used according to a published procedure (Hollender and Liu, 2010). Results were imaged under a Zeiss Axio Observer.Z1 inverted microscope. The experiment was performed twice.

RESULTS AND DISCUSSION

miR172 expression is altered in *lug-3* flowers

lug mutant flowers exhibit partially homeotic transformation of sepals to carpelloid sepals, indicating an expansion of C function into the first whorl (Liu and Meyerowitz, 1995). To test whether miR172, which is expressed in the inner floral whorls (Chen, 2004; Wollmann et al., 2010), expands into *lug* outer whorls, small RNA blots and in situ hybridization were performed. Increased miR172 was detected in *lug-3* flowers (Fig. 1A), consistent with preliminary microarray data showing increased expression of miR172a-e (see Fig. S1 in the supplementary material). This increased miR172 in *lug-3* correlates with a decrease in *AP2* mRNA (Fig. 1B).

In situ hybridization was used to examine miR172 expression in *lug-3* as well as in *seu-1* because SEU is a known partner of LUG (Sridhar et al., 2006). The *miR172* antisense probe was made by T7 transcription from a direct tandem oligonucleotide concatamer (4×) of the sense *miR172* strand. Using a similar probe, miR172 was previously shown to be expressed in the entire floral meristem, but the expression was abated from sepals in stage 7 and older flowers (Chen, 2004). We showed that miR172 RNA is no longer present in the sepals of stage 6 and older wild-type flowers (Fig. 1C and see Table S2 in the supplementary material). By contrast, in *lug-3* and *seu-1* mutant flowers, miR172 expression was detected in the sepals of stage 6 or older flowers (Fig. 1E,F). This ectopic miR172 expression was, however, sometimes found in only one of the two sepals (Fig. 1E,F). Further quantification revealed that 82% of the *lug-3* (14 of 17) or *seu-1* (18 of 22) sepals at stages 6 to 11 showed ectopic miR172 expression (see Table S2 in the supplementary material). This correlates well with the incomplete sepal-to-carpel transformations in *lug-3* and *seu-1* mutants (Franks et al., 2002; Liu and Meyerowitz, 1995). The in situ data indicate that *LUG* and *SEU* are each required to repress *miR172* expression in the sepals starting no later than stage 6.

A recent publication using a locked nucleic acid (LNA)-based probe revealed inner whorl-specific miR172 expression as early as floral stage 4 (Wollmann et al., 2010). This difference in the timing (i.e. stage) of miR172 RNA clearing in wild-type sepals might reside in the greater specificity and sensitivity of the LNA probe, which consequently is likely to detect specific miR172 species. We thus focused our analyses of miR172 expression on stage 6 and older sepals, for which both probe types detect a clearing of the miR172 signal in wild type.

LUG directly regulates *miR172*

The increased and ectopic miR172 expression observed above could result from ectopic carpelloid organ development in the outer floral whorls of *lug-3* as carpels are known to express high levels of miR172. We therefore conducted ChIP to test whether LUG directly associates with *miR172* chromatin.

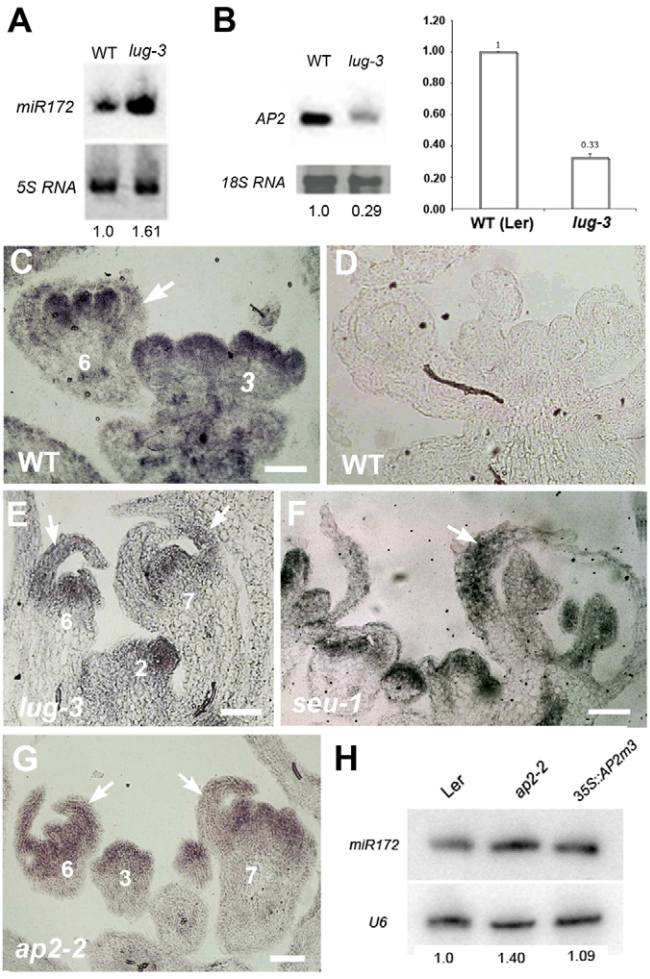

Fig. 1. Increased and ectopic miR172 expression in *lug-3*, *seu-1* and *ap2-2*. (**A**) A small RNA blot showing miR172 RNA levels in *lug-3* and wild-type (WT) *Arabidopsis thaliana* inflorescences. 5S RNA provides the loading control, with the relative signal intensity shown beneath each lane. (**B**) Northern and qRT-PCR showing *AP2* mRNA levels. 18S RNA provides the loading control. Error bars indicate s.d. (**C**) In situ hybridization showing miR172 RNA expression in wild-type flowers. Numbers indicate floral stages. (**D**) *miR172* sense probe in wild type. (**E**) miR172 expression in *lug-3* flowers. Arrows indicate sepals with ectopic miR172 expression. (**F**) miR172 RNA expression in *seu-1* flowers. (**G**) miR172 expression in *ap2-2*. (**H**) A small RNA blot showing miR172 expression in *ap2-2* and *35S:AP2m3* flowers. *U6* provides a loading control. Scale bars: 50 μm.

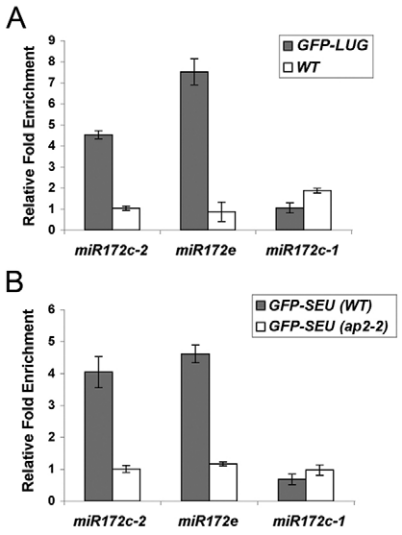

Fig. 2. ChIP detects the in vivo association of GFP-LUG and GFP-SEU with *miR172* promoter sequences. (**A**) ChIP assays with anti-GFP antibody and chromatin isolated from *35S:GFP-LUG; lug-16* (gray) and non-transgenic wild-type (white) *A. thaliana* inflorescences. Fold enrichment was quantified by qPCR using primers that flank putative AP2 binding sites. Error bars indiate s.d. of six replicates (two biological replicates with three technical repeats). (**B**) ChIP assays using anti-GFP antibody and chromatin from *ProSEU:GFP-SEU* in wild type (gray) and *ap2-2* (white). Fold enrichment for the same primer sets as in A is shown. Error bars indiate s.d. of three technical replicates of one biological experiment.

We chose *miR172c* and *miR172e*, which showed the highest increase in expression in *lug-3* mutants (see Fig. S1 in the supplementary material). We searched *miR172c* and *miR172e* promoters for conserved binding sites for class A genes (i.e. MADS box and AP2 domain). No MADS box binding site (CArG box) was found, but four putative AP2 binding sites (TTTGTT; T. Dinha and X.C., unpublished) were found in the promoter of *miR172c* and one in the promoter of *miR172e*. PCR and qPCR primers were designed to flank these putative AP2 binding sites (see Tables S3 and S4 in the supplementary material).

Nuclear extracts of inflorescences from *35S:GFP-LUG; lug-16* and negative control (non-transgenic wild type) were immunoprecipitated with an anti-GFP antibody. The immunoprecipitated DNA was first quantified by semi-quantitative PCR, testing a larger number of primers (see Fig. S2 and Table S3 in the supplementary material). qPCR was subsequently used to test the most relevant promoter regions of target genes (Fig. 2A). For *miR172c*, only binding site *miR172c-2* showed over 4-fold enrichment with the *GFP-LUG; lug-16* sample (Fig. 2A). The other three potential AP2 binding sites, including *miR172c-1*, were not enriched (Fig. 2A and see Table S3 in the supplementary material). For *miR172e*, the single putative AP2 binding site was enriched over 7-fold with the *GFP-LUG; lug-16* sample (Fig. 2A). Neither *miR172c* nor *miR172e* was enriched with the negative control sample (Fig. 2A). A non-target control, *E1F4a1*, showed no enrichment (see Fig. S2 and Table S3 in the supplementary material). These data suggest that AP2, or other AP2 family members, might be involved in recruiting LUG to the promoters of *miR172c* and *miR172e.*

Further evidence of AP2 mediating the repression of *miR172* by LUG/SEU

If AP2 recruits LUG/SEU to repress *miR172*, *ap2* mutants should exhibit expanded expression of miR172 similar to *lug* and *seu* mutants. In situ hybridization revealed ectopic miR172 expression in stage 6 and 7 *ap2-2* sepals (Fig. 1G); 20 out of 20 *ap2-2* sepals at floral stages 6-11 ectopically expressed miR172 (see Table S2 in the supplementary material). This was supported by northern blots (Fig. 1H) showing increased miR172 in *ap2-2* flowers. By contrast, plants that express a miR172-resistant *AP2* (*35S:AP2m3*) did not show reduced miR172 (Fig. 1H). Perhaps an overproliferation of stamens, a miR172-expressing organ, in these *35S:AP2m3* lines (Zhao et al., 2007) compensates for a reduction of miR172.

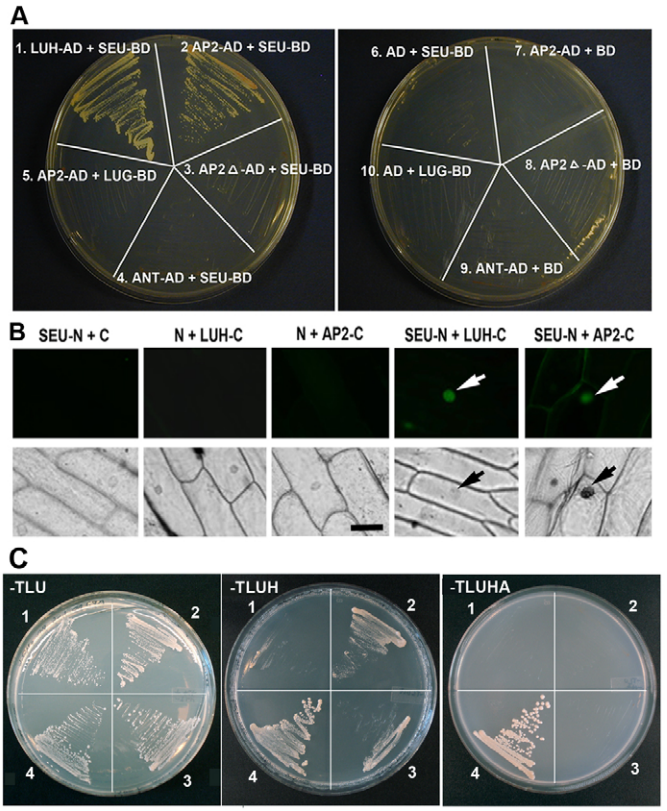

Fig. 3. AP2 interacts with SEU but not LUG. (**A**) A yeast two-hybrid assay between prey (AD) and bait (BD) pairs. LEUNIG HOMOLOG (LUH)-AD against SEU-BD served as a positive control (Sitaraman et al., 2008). SEU-BD contains a truncated SEU (residues 1-563) with its C-terminal domain removed to avoid self-activation (Sridhar et al., 2006). The plate on the right shows various negative controls. The selection medium was –Trp, –Leu, –His, –Ade (–TLHA), plus 3 mM 3-amino-1,2,4-triazole. (**B**) A BiFC assay showing interactions in planta. Fluorescent (top row) and bright-field (bottom row) images of onion epidermal peels bombarded with BiFC plasmids. N and C represent the N- and C-terminal fragments of YFP, respectively. White arrows indicate fluorescent nuclei and black arrows indicate the same nuclei in bright field. SEU-N and LUH-C, previously shown to interact via BiFC (Hollender and Liu, 2010), serve as the positive control. Scale bar: 100μm. (**C**) AP2-AD and LUG-BD were tested for interaction in the presence or absence of full-length SEU expressed from the p426 vector. 1, AP2-AD + p426 vector + LUG-BD; 2, AP2-AD + p426-SEU + LUG-BD; 3, AP2delta-AD + p426-SEU + LUG-BD; 4, SEU-AD + p426 + LUG-BD (positive control). The '–TLU' medium, which lacks Trp, Leu and urea, selects for all three plasmids. '–TLUH' selects for His3. '–TLUHA' selects for His3 and Ade2.

As *GFP-LUG (ap2-2)* plants are not currently available, *GFP-SEU (ap2-2)* plants were used for ChIP analysis to test the role of AP2 in mediating the association of the LUG/SEU co-repressors with *miR172c* and *miR172e* (Fig. 2B). *GFP-SEU (WT)* and *GFP-SEU (ap2-2)* inflorescences were analyzed by ChIP using the anti-GFP antibody. The same qPCR primer pairs as in Fig. 2A were used for quantifying fold enrichment. *miR172c-2* and *miR172e* were enriched 4- and 4.8-fold, respectively, with *GFP-SEU (WT)* but not with *GFP-SEU (ap2-2)* (Fig. 2B). These data support the contention that AP2 is required for mediating the association of SEU with the *miR172c-2* and *miR172e* promoters. Similar to GFP-LUG (Fig. 2A), GFP-SEU failed to associate with *miR172c-1* (Fig. 2B). Since GFP-LUG and GFP-SEU are both associated with the same AP2 binding sites (*miR172c-2* and *miR172e*) out of those tested (Fig. 2A,B and see Table S3 in the supplementary material), it suggests that LUG and SEU might function together in a complex, tethered by AP2, to regulate *miR172*. Using ChIP-seq, Yant et al. (Yant et al., 2010) reported the binding of AP2 to *miR172b* but not to other *miR172* genes. This difference might reside in the different developmental stages at which the tissues were collected in the two studies.

SEU mediates the interaction between AP2 and LUG

The above results suggest that AP2 might directly interact with SEU and/or LUG. A yeast two-hybrid assay revealed a direct interaction of AP2-AD with SEU-BD (Fig. 3A, sector 2) but not with LUG-BD (Fig. 3A, sector 5). A truncated AP2 (AP2delta) containing the two AP2 domains (residues 124-394) failed to interact with SEU-BD (Fig. 3A, sector 3). Thus, the two AP2 domains are not sufficient for the interaction with SEU. Another AP2 domain-containing protein, AINTEGUMENTA (ANT), failed to interact with SEU (Fig. 3A, sector 4), indicating that SEU specifically interacts with AP2.

Bimolecular fluorescence complementation (BiFC) was used to confirm the interaction between SEU and AP2 via particle bombardment in onion cells. SEU and AP2 were each fused to the N-terminal and C-terminal fragments of YFP using the pUC-SPYNE and pUC-SPYCE vectors, respectively (Walter et al., 2004). The fusion proteins SEU-N and AP2-C were observed to interact in nuclei, whereas the negative controls did not (Fig. 3B).

Since LUG is known to interact with SEU (Sridhar et al., 2004), SEU might bridge the interaction between LUG and AP2. A yeast three-hybrid assay was employed to test whether LUG-BD interacts with AP2-AD in the presence or absence of SEU (Fig. 3C). The '–TLUH' and '–TLUHA' media represent two different selection stringencies. AP2-AD interacted with LUG-BD only when SEU was present (Fig. 3C, sector 2) and only in the less stringent –TLUH medium. AP2-AD failed to interact with LUG-BD when SEU was absent (sector 1). AP2delta-AD weakly interacted with LUG-BD in the presence of SEU (sector 3). The data support the proposal that SEU is capable of bridging the interaction between AP2 and LUG. The weakness of the interaction might reflect that all three proteins have to be expressed at the same time and in the same cellular compartments. This result is consistent with synergistic and semi-dominant genetic interactions among *lug*, *seu* and *ap2* mutants during floral organ development (Franks et al., 2002; Liu and Meyerowitz, 1995), indicating functional interactions in vivo.

A model summarizing current understanding of A-C antagonism

miR172 regulates many important developmental processes in diverse plant species (Aukerman and Sakai, 2003; Chen, 2004; Chuck et al., 2007; Jung et al., 2007; Lauter et al., 2005; Martin et al., 2009; Wu et al., 2009). Whereas much attention has focused on finding the targets of miRNAs and on their biogenesis and metabolism, less is known about their transcriptional regulation. Our analysis provides important insights into the domain-specific transcriptional regulation of *miR172*.

Integral to the ABCE model is the mutual antagonism between the A and C class genes. The repression of A class activity in inner whorls is conferred by at least two mechanisms: the post-transcriptional downregulation of *AP2* by miR172 (Fig. 4a) and the repression of *AP1* transcription by AG (Fig. 4b) (Aukerman

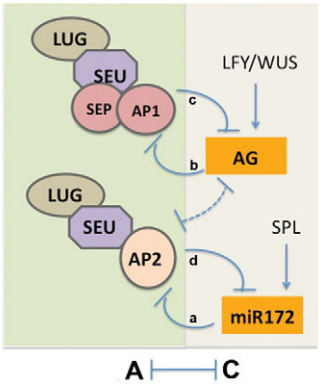

Fig. 4. Model of A and C class gene antagonism. The negative regulation of class A gene activity in the inner two whorls is conferred by the post-transcriptional downregulation of *AP2* by miR172 (a) and by the transcriptional repression of *AP1* by AG (b). By contrast, the negative regulation of the class C gene *AG* in the outer two whorls depends on the LUG/SEU co-repressors recruited by the AP1/SEP MADS box proteins (c). The repression of *miR172* also requires LUG/SEU and is mediated by AP2 (d). The dashed line represents a less well understood regulatory interaction between *AG* and *AP2*. Also indicated are the transcriptional activation of *AG* by LEAFY (LFY) and WUSCHEL (WUS) (Lohmann et al., 2001) and the activation of *miR172* by SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) (Wu et al., 2009).

and Sakai, 2003; Chen, 2004; Gustafson-Brown et al., 1994; Schwab et al., 2005). However, AG and miR172 appear to act independently of each other (Wollmann et al., 2010; Zhao et al., 2007). The repression of class C genes in the outer whorls depends on the LUG/SEU co-repressors recruited by the AP1/SEP3 MADS box proteins (Sridhar et al., 2006) (Fig. 4c) as well as by AP2 (Fig. 4d). The mutual repression between AP2 and miR172 ensures positive-feedback loops that promote their own expression in the respective A and C domains (Fig. 4). It remains unresolved as to how the A-C boundary is initially established. A strict temporal order of gene activation might be one mechanism, so that earlier-acting class A genes are subsequently switched off in the inner whorls once the class C genes are switched on.

Transcriptional co-repressors are known to regulate diverse targets and developmental pathways and their target specificity is conferred by DNA-binding partners (Liu and Karmarkar, 2008). This work has reported a new regulatory target, *miR172*, of LUG/SEU and identified AP2 as the DNA-binding partner of LUG/SEU for *miR172* regulation, providing novel insights into co-repressor function in plant development.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.058362/-/DC1

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Table S1. Probe and primer sequences (5′ to 3′) used in RNA blots, qRT-PCR and cloning

microRNA	Probe sequence	
miR172	CTGCAGCATCATCAAGATTCT	
Primer	To prepare northern probes	
AP2-F	GACGCACCACACCAAACACA	
AP2-R	ATCTTGGTCCACGCCGACTC	
5.8S rRNA-F	GCGCAACTTGCGTTCAAAGA	
5.8S rRNA-R	TTGTGACACCCAGGCAGACG	
Primer	qRT-PCR of *AP2*	Annealing
AP2-qRT-F	CGAAGCTGCTAGAGCTTACG	57°C
AP2-qRT-R	CGAGGTTGTGATCTTGTGGAGTAG	
GAPC-qRT-F	CCAGTCACTGTTTTCGGCATCA	60°C
GAPC-qRT-R	AGCTGCAGCCTTGTCTTTGTCA	
Primer	Cloning for BIFC	Sites added
AP2-cDNA-F	ATGTCGACATGTGGGATCTAAACGACGCA	*Sal*I
AP2 cDNA-R	CCCGGGTCCAGAAGGTCTCATGAGAGGAG	*Xma*I
SEU-cDNA-F	ATGTCGACATGGTACCATCAGAGCCGCCTAAT	*Sal*I
SEU-cDNA-R	CCCGGGTCCCGCGTTCCAATCAAAATT	*Xma*I
LUH-cDNA-F	ATGTCGACATGGCTCAGAGTAATTGGGAA	*Sal*I
LUH-cDNA-R	CCCGGGCTTCCAAATCTTTACGGATTTGT	*Xma*I

Table S2. Percentage of sepals that ectopically express miR172

Genotype	Percentage sepals (stages 6-11) that showed miR172 expression*	Total number of sepals at stages 6-11*
WT (L*er*)	8[†]	37
lug-3	82	17
seu-1	82	22
ap2-2	100	20

*Summary of results from three independent in situ experiments. Each experiment examined three to five inflorescences of each genotype. In most cases, two sepals per flower (at stages 6-11) were scored. Flowers older than stage 11 had been removed before sample fixation.
[†]Three of the 37 wild-type sepals appeared to show some miR172 signals, which might represent background signals.

Table S3. Semi-quantitative PCR primers and summary of ChIP results

Gene	PCR primer (5′ to 3′)	AP2 binding site*	Enrichment (LUG-GFP) (*lug-16*)	Enrichment (SEU-GFP) (*WT*)
miR172c-F1	GAGATTACGAGAATCCGCACTCA	1.6 kb	No	No
miR172c-R1	GGTTTTAGGCTTTTAGCCCAAGGA			
miR172c-F2	AGGATCCACATGTGCCCATA	1.4 kb	Yes	Yes
miR172c-R2	CGTGAGCTATTCAGGATCACGA			
miR172c-F3	TCGCTGACTAAATAGTTGGA	366 bp	No	No
miR172c-R3	GAGGCCAATTTTGTCATCC			
miR172c-F4	ACCTGAGTATCTGAGATCTCAGT	225 bp	No	No
miR172c-R4	AGGGATGTATGTAGTGATTTGG			
miR172e-F	AGCCTTTGGCTTCTGTTCCTGA	118 bp	Yes	Yes
miR172e-R	CGGTTTCGAGGTCTAAAGTTGGGA			
EIF4a1-F	CGCATCCTATCGGATTGTCT	NA	No	No
EIF4a1-R	CTCAGATGATGTGCGGAGAA.			

*Approximate location of each AP2 binding site from the start of cDNA (*miR172*) based on sequence information from www.arabidopsis.org. NA, not available.

Table S4. qPCR primer sequences and summary of ChIP results

Gene	qPCR primer (5′ to 3′)	AP2 binding site*	Enrichment (LUG-GFP) (*lug-16*)	Enrichment (SEU-GFP) (*WT*)	Slope	Primer percentage efficiency
miR172c-F1	GAGATTACGAGAATCCGCACTCA	1.6 kb	No	No	–3.246	103.2
miR172c-R1	GGTTTTAGGCTTTTAGCCCAAGGA					
miR172c-F2	CCACATGTGCCCATATTGAT	1.4 kb	Yes	Yes	–3.518	92.3
miR172c-R2	GAAGATCCACTTTTAAAGCCCAAT					
miR172e-F	GCTGTCTGAATCCTCTTGCTTTCCTC	118 bp	Yes	Yes	–3.155	107.4
miR172e-R	CGGTTTCGAGGTCTAAAGTTGTGA					

*Approximate location of each AP2 binding site from the start of cDNA (*miR172*) based on sequence information from www.arabidopsis.org.