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On reconciling the interactions between *APETALA2*, miR172 and *AGAMOUS* with the ABC model of flower development

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SUMMARY

The ABC model of flower development explains how three classes of homeotic genes confer identity to the four types of floral organs. In *Arabidopsis thaliana*, *APETALA2* (*AP2*) and *AGAMOUS* (*AG*) represent A- and C-class genes that act in an antagonistic fashion to specify perianth and reproductive organs, respectively. An apparent paradox was the finding that *AP2* mRNA is supposedly uniformly distributed throughout young floral primordia. Although miR172 has a role in preventing AP2 protein accumulation, miR172 was reported to disappear from the periphery only several days after *AG* activation in the center of the flower. Here, we resolve the enigmatic behavior of *AP2* and its negative regulator miR172 through careful expression analyses. We find that *AP2* mRNA accumulates predominantly in the outer floral whorls, as expected for an A-class homeotic gene. Its pattern overlaps only transiently with that of miR172, which we find to be restricted to the center of young floral primordia from early stages on. MiR172 also accumulates in the shoot meristem upon floral induction, compatible with its known role in regulating *AP2*-related genes with a role in flowering. Furthermore, we show that *AP2* can cause striking organ proliferation defects that are not limited to the center of the floral meristem, where its antagonist *AG* is required for terminating stem cell proliferation. Moreover, *AP2* never expands uniformly into the center of *ag* mutant flowers, while miR172 is largely unaffected by loss of *AG* activity. We present a model in which the decision whether stamens or petals develop is based on the balance between *AP2* and *AG* activities, rather than the two being mutually exclusive.

KEY WORDS: MicroRNA, miRNA, miR172, *APETALA2*, *AGAMOUS*, ABC model, Homeotic genes, *Arabidopsis*

INTRODUCTION

Flower formation in plants requires the establishment of four types of floral organs arranged in concentric whorls: the sepals and petals, which comprise the sterile perianth; and the stamens and carpels, which are the male and female reproductive organs. The ABC model, first proposed two decades ago, describes how the combinatorial interaction of three classes of homeotic genes directs the development of floral organs (Bowman et al., 1991; Coen and Meyerowitz, 1991). According to this classical model, *Arabidopsis thaliana* A-class genes *APETALA1* (*AP1*) and *AP2* confer sepal identity in the first floral whorl. Their activity overlaps with B-class genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) in the second whorl, which develops into petals. *AP3*, *PI* and the C-class gene *AGAMOUS* (*AG*) specify stamen identity in whorl three, while *AG* alone in whorl four promotes carpel development. The ABC model was initially deduced from loss-of-function effects. Subsequent cloning of the ABC genes showed that *AP1*, *AP3*, *PI* and *AG* all encode MADS domain proteins, as do the *SEPALLATA* (*SEP*) genes, which encode obligatory co-factors for the homeotic proteins.

An essential postulate of the ABC model is the antagonistic and mutually exclusive action of A and C function genes. In *ap2* mutant flowers, expanded *AG* activity leads to the development of reproductive organs at the floral periphery. Conversely, *ag* mutants show transformation of reproductive into perianth organs, an expansion of A function towards the center of the flower. According to the ABC model, A-class function in *Arabidopsis* is, therefore, required for perianth identity and repression of C-class function. Genes with such dual A function have, however, not yet been found in any other species, questioning the generality of A-class function and its role in determining perianth identity (Causier et al., 2010).

In contrast to the highly specific expression of MADS box floral homeotic genes, it has been reported that *AP2* mRNA accumulates not only in the perianth, but also in reproductive organ primordia. Three independent groups have suggested that primary *AP2* expression and promoter activity occur throughout all floral whorls (Jofuku et al., 1994; Würschum et al., 2006; Zhao et al., 2007). A fourth study agreed that *AP2* is expressed ubiquitously, but with transiently stronger mRNA accumulation in different organ primordia (Alvarez-Venegas et al., 2003). Broad expression has been reported for an apparent *AP2* ortholog in petunia (Maes et al., 2001), whereas *AP2* orthologs in snapdragon and in maize have very specific expression patterns in inflorescences and floral primordia (Chuck et al., 1998; Keck et al., 2003).

Apart from its role in specifying floral organ identity, *AP2* can promote ectopic organ formation, an activity that depends at least in part on the stem cell factor WUSCHEL (WUS) (Chen, 2004; Zhao et al., 2007). In flowers, WUS is a co-activator of *AG* expression during early stages of development, while repression of *WUS* by *AG* at later stages is required to produce determinate flowers (Lenhard et al., 2001; Lohmann et al., 2001). Similar to *wus* mutations, a dominant-negative allele of *AP2* has been reported to cause precocious termination of the shoot apical meristem, in support of a positive effect of *AP2* on *WUS* that is independent of its negative role in *AG* regulation (Würschum et al., 2006).

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AP2 expression is regulated at the post-transcriptional level by a microRNA (miRNA), miR172 (Aukerman and Sakai, 2003; Chen, 2004; Kasschau et al., 2003; Rhoades et al., 2002). Transcript cleavage and translational inhibition both play a role in *AP2* regulation by miR172, although assessing the relative importance of the two processes is confounded by a negative-feedback loop in which AP2 represses its own transcription (Aukerman and Sakai, 2003; Chen, 2004; Kasschau et al., 2003; Mlotshwa et al., 2006; Schwab et al., 2005). The discovery of miR172 as post-transcriptional negative regulator of *AP2* immediately provided a potential means to solve the apparent paradox of *AP2* mRNA being ubiquitously expressed, yet repressing *AG* only in the outer two floral whorls. However, miR172 expression was reported to overlap extensively with *AP2* mRNA throughout young floral primordia, and to disappear from the periphery only during stage 7, long after *AG* is activated (Chen, 2004). Thus, miR172-guided regulation alone does not suffice to explain the paradoxical relationship between *AP2* expression and its genetic activity.

Here, we have re-examined not only *AP2* mRNA expression, but also the pattern of miR172 accumulation using in situ hybridization with LNA (locked nucleic acid) probes. We find that upon floral induction, miR172 is strongly upregulated in the shoot meristem, where it has not been observed before (Chen, 2004). In young floral primordia, its expression pattern closely resembles that of *AG*, being mostly concentrated in the floral center. We also find *AP2* to be expressed much more specific, accumulating predominantly in the periphery of floral primordia, with only limited overlap to miR172. We further show that these expression patterns of *AP2* and miR172 are required for proper flower development.

MATERIALS AND METHODS

Plant material

Plants were grown in long-day (16 hours light and 8 hours dark) or short-day (8 hours light and 16 hours dark) conditions at 23°C and 65% humidity. *Arabidopsis thaliana* Col-0 and L*er*-1 plants were used as wild type. *ag*-1 (Bowman et al., 1989), *ag*-2 (Yanofsky et al., 1990) and *dcl1*-11 (renamed from *dcl1*-100) (Laubinger et al., 2008) have been described. The *ap2* allele was obtained from the Salk T-DNA collection (Salk_071140) (Alonso et al., 2003) and was named *ap2-12* (Yant et al., 2010).

In situ hybridization

Tissue was harvested into FAA solution (3.7% formaldehyde, 50% ethanol, 5% acetic acid). For embedding, an automated system (Advanced Smart Processor ASP300, Leica, Wetzlar, Germany) was used. Sections of 8 or 9 μm thickness were prepared using a rotary microtome (Leica RM2165). Hybridization and detection were carried out as described (Palatnik et al., 2003) with some modifications. After incubation in Histoclear, the sections were processed through an ethanol series, treated with Proteinase K (Roche) for 30 minutes at 37°C and post-fixed with FAA. Hybridization was carried out at 55°C overnight. Slides were blocked with 1% blocking reagent (Roche, Mannheim, Germany) in 1×TBS/0.3% Triton X-100. For immunological detection, anti-DIG antibody (Roche) was used in a 1:1259 dilution. NBT/BCIP stock solution (Roche) for color reaction was diluted 1:50 in 10% polyvinyl alcohol (PVA) in TNM-50. Probes were synthesized with the DIG RNA Labeling Kit (Roche) on PCR products of the target genes. For the *AP2* (At4g36920) 3′ end probe, a 634 bp cDNA fragment was PCR amplified and cloned into pBluescript (pHW083). Oligonucleotide sequences are listed in Table S1 in the supplementary material. The *AG* (At4g18960) and *WUS* (At2g17950) probes were based on previously described plasmids (Leibfried et al., 2005; Yanofsky et al., 1990). The miR172 antisense LNA (locked nucleic acid, Exiqon, Vedbaek, Denmark) oligonucleotide with the sequence atg^{m}Cag^{m}Cat^{m}Cat^{m}CaaGatTct (upper case, LNA; lower case: DNA) was end-labeled with the DIG 3′-End Labeling Kit (Roche) and purified with Micro Spin Chromatography Columns (Bio-Rad, Hercules, CA, USA). LNA-based miRNA in situ hybridization was carried out largely according to the same procedure. Proteinase K incubation was carried out for 25 minutes at 37°C. For post-fixation, 4% (w/v) paraformaldehyde in 1× PBS was used. After washing, the slides were incubated in 0.1 M triethanolamine (pH 8.0) and 0.5% acetic anhydride for 10 minutes. RNase treatment was carried out after hybridization and slides were prepared for immunological detection by 45-minute incubation each in 0.5% blocking reagent (Roche) and buffer B (1% BSA, 0.3% Triton X-100 in 1×TBS); the latter was used also for subsequent washing steps. In an independent line of experiments, using the protocol of (Long and Barton, 1998), an *AP2* full-length probe was used to detect *AP2* expression in plants of the Landsberg *erecta* (L*er*-1) background.

Cloning and transgenic plants

The binary plasmids are listed in Table 1. Oligonucleotide primer sequences for PCR amplification and PCR-based mutagenesis are listed in Table S1 in the supplementary material. For the *pAP2:AP2::YFP* reporter, two copies of the coding sequence of yellow fluorescent protein for energy transfer YPet were fused in frame with the C terminus of *AP2* in the JAtY57F17 TAC (transformation-competent artificial chromosome) clone (Liu et al., 1999), which is ~32 kb in length, using a bacterial recombineering approach (Warming et al., 2005). For the *pAP2:AP2::GUS* reporter, an ~5 kb upstream fragment and the *AP2* transcribed region were amplified with primers that included sequences for recombination using the Gateway technology (Invitrogen, Carlsbad, CA, USA). The *AP2* promoter and the *AP2* transcribed region from ATG to the stop codon were recombined into pDONR P4-P1R (Invitrogen) and pDONR/Zeo (Invitrogen), respectively. The β-*glucuronidase* (GUS) gene was introduced into pDONR P2R-P3 (Invitrogen). The three inserts were combined into a pALLIGATOR2 binary plasmid (Bensmihen et al., 2004) (http://www.isv.cnrs-gif.fr/jg/alligator/vectors.html) that was modified to allow MultiSite Gateway (Invitrogen) recombination. Primary transgenic plants were selected based on GFP fluorescence of dry seeds. Other binary plasmids were based on pGreenII (Hellens et al., 2000) and modified to allow Gateway (Invitrogen) compatible cloning. CaMV35S and *AP3* promoter sequences were as described (Lohmann et al., 2001). The wild-type and miR172 targeting resistant (*rAP2*) versions of *AP2* have been described (Schwab et al., 2005), and were also introduced into Gateway compatible entry plasmids. The artificial target mimicry construct *MIM172*

Table 1. Plant transformation vectors

Plasmid number	Description	Purpose
HW075	p35S (empty)	Control transgenic plants
HW216	pAP3:MIM172	Region-specific miR172 knock-down
HW230	pAP3:AP2	Region-specific wild-type *AP2* mis-expression
HW245	pAP3:rAP2	Region-specific miR172 resistant *AP2* mis-expression
HW235	pAP3:amiR-AP2	Region-specific *AP2* knock-down
HW210	p35S:amiR-AG-1	Broad *AG* knock-down
HW209	pAP3:amiR-AG-1	Region-specific *AG* knock-down
HW222	p35S:amiR-AG-2	Broad *AG* knock-down
HW319	pAP2:AP2::GUS	AP2 genomic reporter
JAS100	pAP2:AP2::YFP	AP2 genomic reporter

was generated by PCR-based mutagenesis of *IPS1* (Franco-Zorrilla et al., 2007). Artificial miRNAs were designed using WMD (http://wmd2.weigelworld.org) (Ossowski et al., 2008).

Microscopy

For scanning electron microscopy, single flowers from T1 transgenic lines were fixed for 5 minutes in 100% methanol and then transferred to ethanol. After critical point drying and coating with gold and palladium 30 nm particles, samples were examined using a Hitachi S800 electron microscope.

For the *pAP2:AP2::YFP* reporter, whole inflorescences were embedded in 3% agarose and placed in a chambered coverglass (NUNC, Rochester, NY) for imaging on a Leica DM IRE2 laser-scanning confocal microscope.

RESULTS

Patterns of *AP2* and miR172 expression in shoots and flowers

AP2 is one of the four genes in the original ABC model of floral organ specification (Bowman et al., 1991; Coen and Meyerowitz, 1991). In contrast to the other three genes, *AP3*, *PI* and *AG* (Drews et al., 1991; Goto and Meyerowitz, 1994; Jack et al., 1994), as well as the other A function gene *AP1* (Mandel et al., 1992), its reported broad mRNA expression pattern during early floral development does not correlate well with its role in conferring specifically perianth identity. MiR172 negatively regulates *AP2*, but its reported distribution throughout all whorls until floral stage 6 (Chen, 2004) does not satisfactorily explain the discrepancy between *AP2* mRNA expression and its specific activity. We therefore decided to re-examine the localization of miR172 and *AP2* transcripts specifically during early flower development.

Because the *MIR172a-2* precursor has been shown to be transcriptionally upregulated at the shoot apex upon photoperiodic induction of flowering (Schmid et al., 2003), we chose vegetative and inflorescence apices during the transition to flowering to establish locked nucleic acid (LNA)-based in situ hybridization for detection of miR172. Although the miR172 signal was low in vegetative apices of 3-week-old, short-day grown plants, it appeared within 1 day of the transfer to long days, which induces flowering. It further increased during days 3 and 5, when the first signs of inflorescence elongation became apparent (Fig. 1A).

Next we looked at miR172 expression in early floral primordia. Based on a different in situ hybridization approach, it has been reported that miR172 expression is absent from the shoot meristem, that it is abundant in stage 1 floral primordia and that it persists in all four floral whorls through stage 6 of flower development (Chen, 2004). Using the LNA-based method, however, we found miR172 expression to be at higher levels in the shoot apical meristem than in stage 1 and 2 flower primordia (Fig. 1B). From stage 3 onwards, we observed graded miR172 expression that was highest in the center of the floral meristem, which gives rise to the fourth whorl (Fig. 1C,D). The miR172 signal persisted in the fourth whorl the longest, while it was low or absent in the other floral whorls (Fig. 1E,F). Expression became restricted to the base of the developing gynoecium, and was subsequently detected in developing ovules (Fig. 1F,G). This last expression pattern might be related to the role of the miR172 target *AP2* in integument development (Léon-Kloosterziel et al., 1994; Modrusan et al., 1994). Because the expression of miR172 in the center of developing flowers from stage 3 onwards is similar to that of *AG* (Drews et al., 1991), we asked whether *AG* is required for maintenance of the proper miR172 pattern. In *ag*-2 mutant flowers, early miR172 expression was similar to its pattern in wild type (Fig. 1H-J), but persisted in the indeterminate floral meristem (Fig. 1K,L).

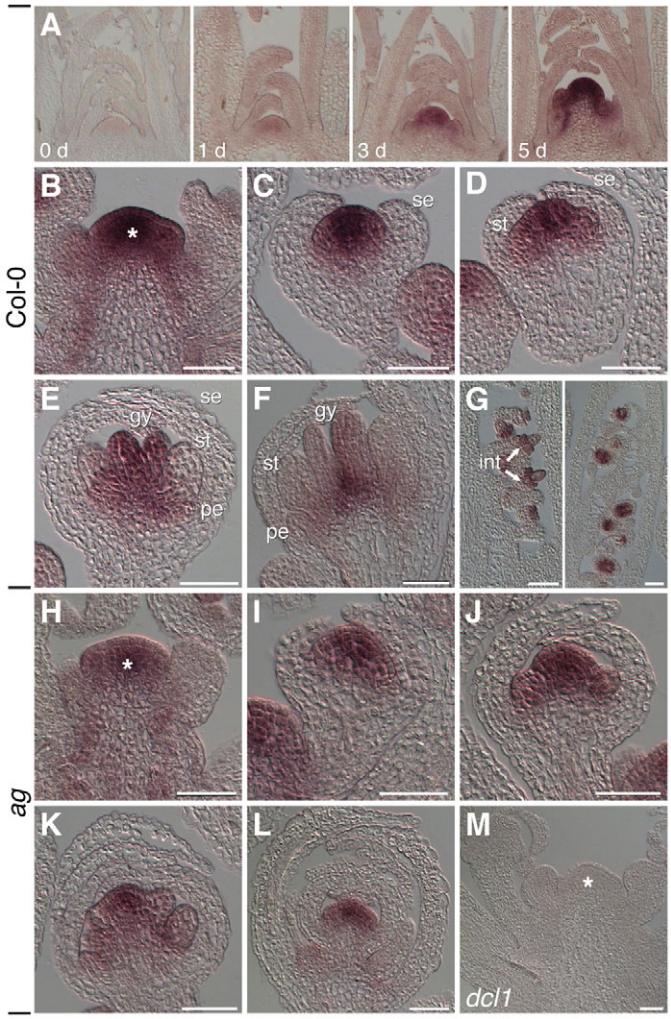

Fig. 1. Expression of miR172. (**A-G**) Col-0 wild type. (A) Apices from plants grown in short days and transferred to long days to induce flowering. Days after shift are indicated at the bottom. (B) Inflorescence meristem (asterisk) with flanking stage 1 and 2 floral primordia. (C) Stage 4 flower. (D) Stage 5 flower. (E) Stage 7 flower. (F) Stage 8 flower. (G) Developing ovules with signal in integuments. (**H-L**) *ag*-2. (H) Inflorescence meristem (asterisk) with flanking stage 1 and 2 floral primordia. (I) Stage 5 flower. (J) Stage 6 flower. (K) Approximately stage 7 flower. (L) Later stage flower. (**M**) *dcl1*-11 inflorescence apex (asterisk). se, sepal; pe, petal; st, stamen; gy, gynoecium; int, integuments. Scale bars: 50 μm.

As a negative control, we performed in situ hybridization on plants with a strong hypomorphic allele of *DICER LIKE1* (*DCL1*), the Dicer responsible for miRNA biogenesis in *A. thaliana* (Park et al., 2002). No miR172 signal was detected (Fig. 1M).

We complemented the in situ hybridization studies of miR172 with analyses of its target *AP2*, using a probe against the 3′ region of the transcript to avoid cross hybridization with homologs. In contrast to previous reports (Jofuku et al., 1994; Würschum et al., 2006), we found a distinct accumulation pattern of *AP2* mRNA throughout reproductive development (Fig. 2; see Fig. S1 in the supplementary material). In Col-0 wild-type inflorescences, strong *AP2* signal was detected in floral primordia from the earliest stages on. It became rapidly restricted to the periphery from stage 2 onwards (Fig. 2A). During stage 3, *AP2* signal was abundant in sepals emerging on the flanks of the floral primordia (Fig. 2B,C).

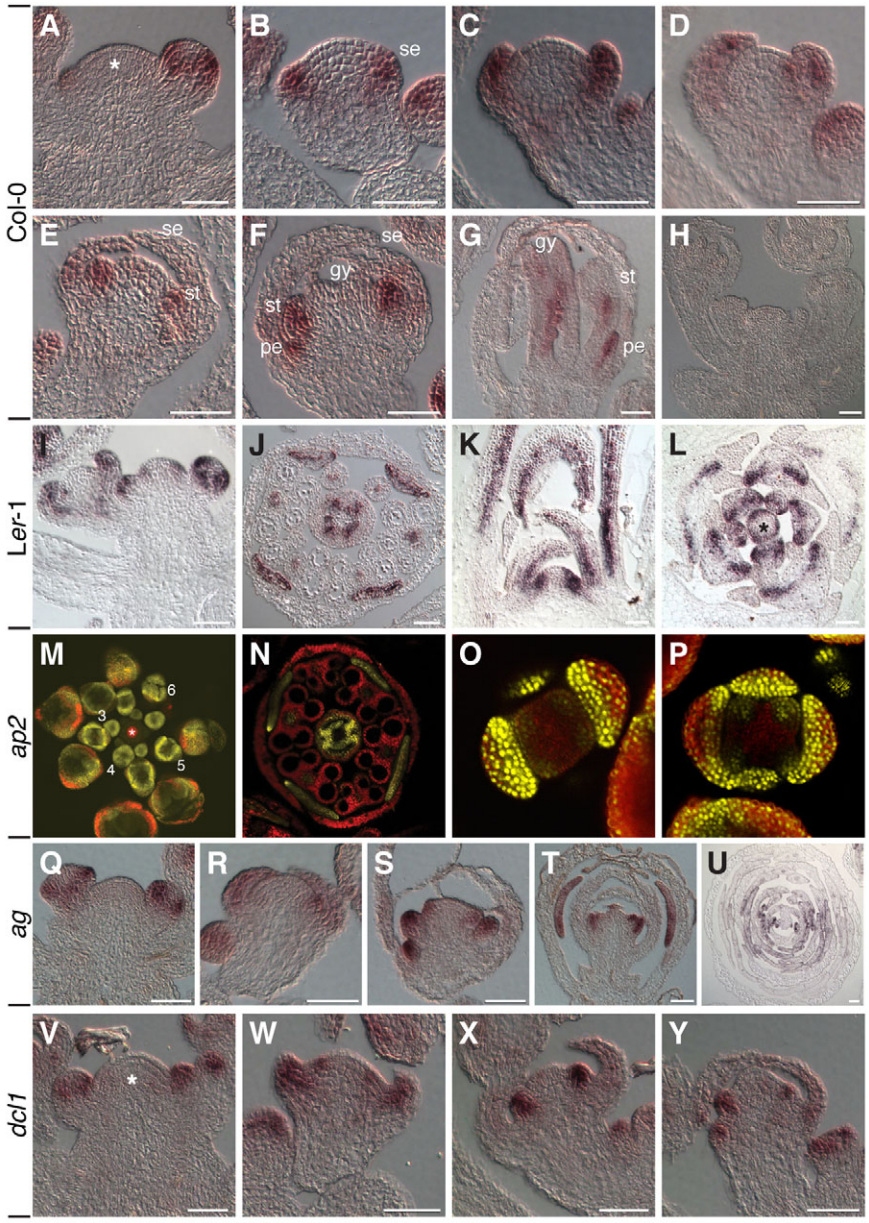

Fig. 2. Expression of *AP2*. (**A-H**) Col-0 wild type. (A) Inflorescence meristem (asterisk), with flanking stage 1 and 2 floral primordia. (B) Stage 3 flower. (C) Late stage 3 flower. (D) Stage 4 flower. (E) Stage 5 flower. (F) Stage 6 flower. (G) Stage 9 flower. Expression of *AP2* is present in petals, stamen filaments and placenta with developing ovules. (H) Inflorescence apex hybridized with sense probe. (**I,J**) L*er*-1 wild-type inflorescence apex (I) and cross section through an approximately stage 12 flower (J). (**K**) Longitudinal section of vegetative L*er*-1 apex. (**L**) Transverse section. *AP2* expression is found in emerging leaf primordia on the flanks of the shoot apical meristem (asterisk). In developing leaves, *AP2* expression is strongest laterally and adaxially. (**M-P**) Transgenic plants carrying a *pAP2:AP2::YFP* reporter. Entire inflorescence (M), cross-section through an approximately stage 12 flower (N), and higher magnification of stage 4 (O) and 5 (P) flowers. There is strong YFP signal (yellow) in the sepals from stage 4 flowers onwards (M,O-P) and in stamens and petals (M-P), recapitulating the in situ hybridization pattern (J). In M, numbers indicate floral stages, the asterisk indicates the inflorescence meristem. Background fluorescence is red (M-P). (**Q-T**) *ag*-2. (Q) Inflorescence meristem, with flanking stage 2 and 3 floral primordia. (R) Late stage 4 flower. (S) Approximately stage 7 flower. (T) Late stage with several extra whorls of organs. Expression in petals. (**U**) Cross-section through mature flower of *ag*-1 mutant, with extensive signal in younger petals. (**V-Y**) *dcl1*-11. (V) Inflorescence apex (asterisk). (W) Stage 3 flower. (X) Stage 6 flower. (Y) Later stage. Interior organs develop abnormally. A-H,Q-T,V-Y were hybridized with a probe against the 3′ region of the *AP2* transcript; I-L,U were hybridized with a full-length probe. Description of floral stages follows Smyth et al. (Smyth et al., 1990). se, sepal; p, petal; st, stamen; gy, gynoecium. Scale bars: 50 μm for A-L,Q-Y.

By comparison, *AP2* transcript levels appeared to be low or absent from the shoot apical meristem and the center of floral primordia after stage 2 (Fig. 2A-C). Subsequently, *AP2* signal declined in sepals, but appeared in stamen and petal primordia (Fig. 2D-F). Notably, *AP2* and miR172 signal transiently overlapped in the third, and probably also the second, whorl (Fig. 1C,D; Fig. 2D,E). In later stages of flower development, we observed *AP2* expression in developing petals, stamen filaments and the gynoecium, including placenta and developing ovules (Fig. 2G), consistent with the known role of *AP2* in ovule development (Léon-Kloosterziel et al., 1994; Modrusan et al., 1994). Similar results were obtained with a probe against the full-length *AP2* transcript, which was hybridized to L*er*-1 inflorescences (Fig. 2I,J).

AP2 is closely related to five other genes that encode AP2-type transcription factors and that are also targets of miR172. Four of these have been shown to act as floral repressors (Aukerman and Sakai, 2003; Mathieu et al., 2009; Schmid et al., 2003). A similar role has recently been described for *AP2* (Yant et al., 2010; Mathieu et al., 2009), and vegetative expression of *AP2* has been noted before (Würschum et al., 2006). We used the full-length probe to examine *AP2* expression by in situ hybridization in vegetative tissue. In 25-day-old, short-day grown L*er*-1 apices, *AP2* transcripts were abundant in developing leaves, in particular in adaxial regions (Fig. 2K,L). Additionally, *AP2* appeared to be expressed as a ring around the periphery of the vegetative meristem and to be upregulated in the incipient leaf primordia (Fig. 2L). As a control, we performed in situ hybridization with an *ap2* T-DNA insertion line; much weaker signals were observed with this material (see Fig. S1A-D in the supplementary material).

AP2 levels are regulated by miR172 both through miRNA-guided transcript cleavage and translation inhibition (Aukerman and Sakai, 2003; Chen, 2004), possibly causing AP2 protein localization not to fully overlap with its transcript pattern. We generated two different *AP2* reporter constructs that allowed us to investigate the localization of AP2 fusion proteins. A *pAP2:AP2::GUS* (β-*glucuronidase*) reporter that included ~5 kb of upstream sequences, and the *AP2* transcribed region reproduced several aspects of the *AP2* transcript pattern (see Fig. S1E,F in the

supplementary material), except for the characteristic expression in sepals. We also examined a *pAP2:AP2::YFP* reporter, which was based on an ~32 kb TAC clone and which complemented the *ap2*-2 mutation. This reporter produced strong YFP signal from floral stage 4 onwards in sepal primordia and then in developing sepals, as well as in stamens and petals (Fig. 2M). Later in floral development, YFP signal was observed in petals and the gynoecium, as well as in stamen filaments (Fig. 2N), recapitulating the pattern observed with in situ hybridization (Fig. 2J). Increasing amounts of YFP signal was detected in stamens of stage 4 and 5 flowers (Fig. 2O,P), suggesting that miR172 activity at these stages is not sufficient to fully prevent AP2 protein accumulation. In summary, AP2 protein appears largely to match its transcript localization. Notably, YFP activity was observed in the inflorescence meristem in a subset of plants analyzed, suggesting transient expression that is not easily detected by in situ hybridization.

A central tenet of the ABC model of floral patterning is the mutual antagonism of *AP2* and *AG* (Bowman et al., 1991). We therefore analyzed *AP2* transcripts in *ag* mutant flowers. Although *AP2* expression appeared in the supernumerary floral primordia formed in *ag-2* mutants, it remained below detection level within the meristem itself (Fig. 2Q-T). Similar results were obtained with the full-length probe, which was hybridized to *ag*-1 mutant inflorescences (Fig. 2U).

As we did not detect mature miR172 in *dcl1* mutant flowers (Fig. 1M), these plants also afforded us an opportunity to determine the contribution of miR172 to the spatial pattern of *AP2* mRNA accumulation. Similar to other *dcl1* mutants (Schauer et al., 2002), *dcl1*-11 plants have a broad variety of developmental defects as a result of global reduction in miRNA activity. Therefore, a specific phenotype caused by increased *AP2* activity might be difficult to pinpoint. As in wild type, *AP2* was excluded from the center of the floral and inflorescence meristem (Fig. 2V-Y), but appeared ectopically in the supernumerary organs that developed in *dcl1* mutant flowers during later stages, similar to what we had observed in *ag* mutants (Fig. 2S,Y). We conclude that the low levels of *AP2* mRNA in the center of the flower are largely due to negative factors other than *AG* and miR172, or to the lack of positive factors that activate *AP2* mRNA expression.

Local requirement of miR172 and *AP2* for stamen and petal identity

Although *AP2* transcripts and miR172 accumulated in largely complementary territories, they partially overlapped, particularly during stages 3 to 5 of flower development. To determine the biological significance of miR172-guided *AP2* regulation in this region, we locally knocked down miR172 activity by target mimicry (Franco-Zorrilla et al., 2007). A subset of flowers of T1 transgenic lines expressing the miR172 target mimic from the *AP3* promoter (Jack et al., 1994) suffered from partial loss of stamen identity, normally in one of the two lateral stamens. Affected stamens were incompletely converted into petals (Fig. 3A; see Table S2 in the supplementary material).

In a complementary experiment, we expressed an artificial miRNA (amiRNA) targeting *AP2* under control of the *AP3* promoter (Ossowski et al., 2008; Schwab et al., 2006). We did not observe obvious defects in stamen development, but a minority of T1 transgenic lines showed petal defects, ranging from slightly thinner petals to petals with stamen characteristics at their flanks (Fig. 3B).

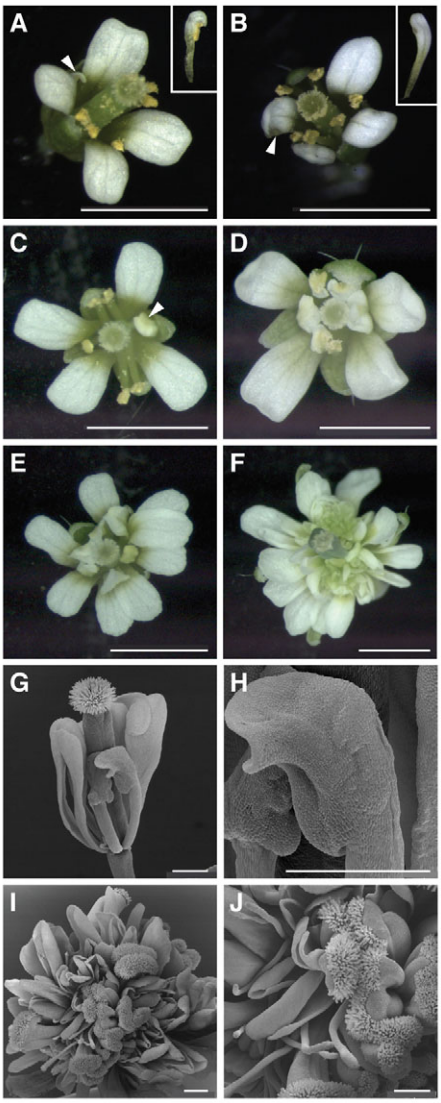

Fig. 3. *pAP3:MIM172*, *pAP3:amiR-AP2* and *pAP3:rAP2* flowers. (**A**) Single flower of a *pAP3:MIM172* transgenic plant; arrowhead indicates a stamen that has been partially converted into a petal, with inset showing higher magnification (see also Table S2 in the supplementary material). (**B**) Single flower of a *pAP3:amiR-AP2* plant. Arrowhead indicates stamenoid tissue on the flanks of a petal, with inset showing higher magnification. Out of 18 T1 plants, four had flowers with slightly abnormal petals. (**C**) Weak *pAP3:rAP2* flower. Arrowhead indicates a petaloid stamen. (**D**,**E**) Intermediate *pAP3:rAP2* flowers. (**F**) Strong *pAP3:rAP2* flower. (**G**) Scanning electron micrograph of a weakly affected *pAP3:rAP2* flower, with perianth partially removed. (**H**) Higher magnification of petaloid stamen. (**I**) Massive organ proliferation in a single old flower of a strong line. (**J**) A higher magnification of I highlighting carpeloid and filamentous organs. Fractions of T1 lines in different phenotypic categories are listed in Table S2 in the supplementary material. Scale bars: 2 mm in A-F; 0.5 mm in G-J.

In summary, region-specific attenuation of miR172 function caused partial stamen-to-petal conversion, while local knockdown of *AP2* activity led to defects suggestive of petal-to-stamen transformation, indicating that local *AP2* action leads to promotion of petal over stamen fate. The genetic evidence thus predicts that miR172 levels are sufficiently high in stamen, but not in petal primordia, to inhibit the function of *AP2*.

Effects of a non-targeted version of *AP2* on organ identity and initiation

An alternative to miRNA target mimicry is the introduction of modified targets that escape miRNA regulation because of silent mutations in the miRNA target site. Transgenic expression of a miR172 non-targeted version of *AP2* (*rAP2*) delays flowering and causes indeterminate growth of flowers with either petal or stamen overproliferation (Chen, 2004; Zhao et al., 2007). To further test the importance of miR172 action for floral patterning of second- and third-whorl floral organs, we expressed an *rAP2* version (Schwab et al., 2005) under the control of the *AP3* promoter. Plants mis-expressing wild-type *AP2* had mostly normal flowers, whereas those mis-expressing *rAP2* often had petaloid stamens (Fig. 3C-E,G,H; see Table S2 in the supplementary material). The most severely affected lines showed complete conversion of stamens into petals (Fig. 3F). The ectopic organs in these lines had petaloid and carpeloid characteristics, the latter forming extensive and partially fused structures with ovules and stigmata (Fig. 3I,J; see Fig. S2A,B in the supplementary material). We also observed filamentous organs, sometimes with stigmatic papillae at their tip (see Fig. S2C,D in the supplementary material). Organ proliferation appeared to mostly be initiated from multiple meristem-like centers within such a flower (see Fig. S2E,F in the supplementary material).

A plausible explanation for the occurrence of supernumerary meristems is ectopic activation of the stem cell factor *WUS*. *WUS* expression from the *AP3* promoter causes ectopic organ formation in the second and third floral whorl (Lohmann et al., 2001), reminiscent of what we observed in severe *pAP3:rAP2* flowers. Because a role for *AP2* in the regulation of *WUS* had been suggested before (Würschum et al., 2006; Zhao et al., 2007), we examined *WUS* expression by in situ hybridization. *WUS* expression persisted longer in the center of *pAP3:rAP2* flowers (Fig. 4). Exact floral stages were difficult to establish, owing to impaired organ development in the second and third floral whorl. In older flowers, we detected ectopic *WUS* expression at the flanks of the delayed carpel that forms in the fourth floral whorl (Fig. 4G-I). In contrast to the wild-type *WUS* pattern, these ectopic patches of expression appeared less well defined and more variable, especially in old flowers (see Fig. S3 in the supplementary material). The abnormal *WUS* expression pattern indicated that the formation of supernumerary organs in severe *pAP3:rAP2* flowers was associated with ectopic meristem activity.

Flowers that constitutively express a non-targeted version of *AP2* initiate extra organs in the central whorl, because of both reduced *AG* activity and ectopic *WUS* function (Zhao et al., 2007). Interestingly, *AG* mRNA expression appeared to be affected most strongly in the center of these flowers, suggesting that *AP2* and *AG* expression were still overlapping (Zhao et al., 2007). However, because *AP2* mRNA expression was not examined, the precise relationship between *AP2* and *AG* patterns in these plants is unknown. To further elucidate the role of *AG* in mediating the effects of ectopic *AP2* activity, we compared *AP2* and *AG* mRNA patterns in flowers of *pAP3:rAP2* plants. As expected, there was strong *AP2* mRNA accumulation in the second and third whorls of floral primordia (Fig. 5A-F). *AG* transcript levels were reduced in the third whorl, where *AP2* was strongly expressed, but appeared largely normal in the central fourth whorl (Fig. 5K-P), indicating that ectopic *AP2* activity in *pAP3:rAP2* plants restricts the *AG* expression domain.

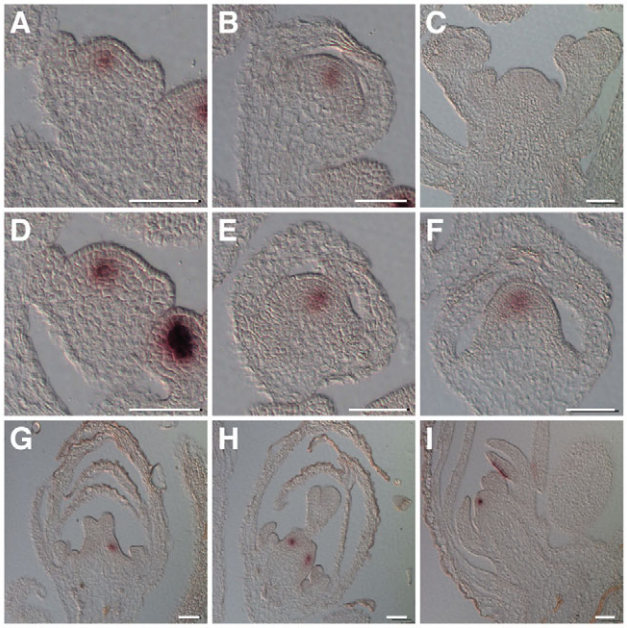

Fig. 4. *WUS* expression in strongly affected *pAP3:rAP2* flowers. (**A-C**) Transgenic control line containing empty vector with CaMV 35S promoter. (A) Stage 3 flower. (B) Stage 5 flower. (C) Sense probe as control. (**D-I**) *pAP3:rAP2*. (D) Stage 3 flower. (E) Stage 5 flower. (F) Approximately stage 6 flower. *WUS* expression persists in the center, and organ formation is delayed in the second and third whorl. (G-I) Later stages, with ectopic foci of *WUS* expression. Scale bars: 50 μm.

Evidence for *AG*-independent effects of *AP2*

Although the constitutive expression of *rAP2* from the CaMV35S promoter causes indeterminacy effects that are often reminiscent of those seen in *ag* mutants (Zhao et al., 2007), *pAP3:rAP2* flowers had distinct defects, with ectopic *WUS* expression and organ formation that was not limited to the center of the flower. We therefore wanted to test whether reducing *AG* activity in the third whorl would have similar consequences. We generated two amiRNAs against *AG* and analyzed their silencing efficacy by broad overexpression under control of the 35S promoter. One amiRNA (*amiR-AG-2*) caused only mild phenotypes (see Fig. S4B in the supplementary material), but the other (*amiR-AG-1*) could produce *ag*-like phenotypes, with petals replacing stamens in the third whorl and typical indeterminate growth in the fourth whorl (Fig. 6A). Different from strong *ag* mutants, fourth-whorl organs enclosing the newly formed flowers often retained carpeloid features (Fig. 6A) (Bowman et al., 1989). In situ localization of *AP2* mRNA in *35S:amiR-AG-1* flowers confirmed similar effects on *AP2* expression as in *ag* mutant flowers (see Fig. S4A in the supplementary material; Fig. 2S,T). We expressed also *amiR-AG-1* under control of the *AP3* promoter. In some of the transgenic lines, we observed different degrees of stamen-to-petal transformation (Fig. 6B). In contrast to *pAP3:rAP2*, none of the lines had ectopic organs in the third whorl, indicating that the decrease of *AG* levels in these plants was not sufficient to activate *WUS* ectopically. Therefore, the effect of *pAP3:rAP2* on *WUS* expression might indeed be *AG*-independent.

DISCUSSION

For two decades, the ABC model has successfully explained the primary genetic principles of floral organ patterning (Bowman et al., 1991; Coen and Meyerowitz, 1991). In addition to

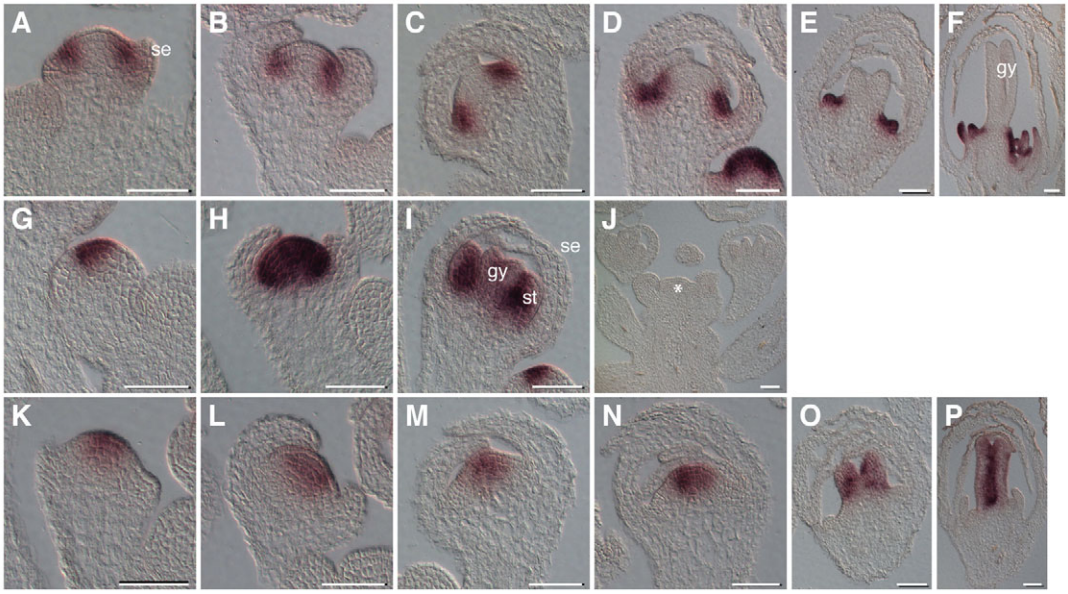

Fig. 5. *AP2* and *AG* expression in strongly affected *pAP3:rAP2* flowers. (**A-F**) *AP2* expression in *pAP3:rAP2*. (A) Stage 3 flower. (B) Late stage 3 flower. (C) Approximately stage 5 to 6 flower. (D-F) Later stages. The endogenous *AP2* expression pattern (Fig. 2) was probably obscured owing to strong activity of the *AP3* promoter. (**G-J**) *AG* expression in transgenic control line containing empty vector with CaMV 35S promoter. (G) Stage 2 flower. (H) Stage 3 flower. (I) Stage 6 flower. (J) Sense probe as control. Asterisk indicates the inflorescence meristem. (**K-P**) *AG* expression in strongly affected *pAP3:rAP2* flowers. (K) Stage 3 flower. (L) Late stage 3 flower. (M) Approximately stage 5 to 6 flower. (N-P) Later stages. se, sepal; st, stamen; gy, gynoecium. Scale bars: 50 μm.

specifying floral organ fate, A and C function genes also restrict each others' action in a mutually antagonistic manner. How this antagonism is achieved has, however, been unclear, as the A-class factor *AP2* has been thought to be expressed throughout floral primordia (Alvarez-Venegas et al., 2003; Jofuku et al., 1994; Würschum et al., 2006; Zhao et al., 2007). Although the discovery of miR172 as a negative regulator was important, it did not appear to be sufficient to explain this discrepancy, because it was reported to be expressed uniformly during the crucial early stages of flower development (Chen, 2004).

Here, we have revealed that neither *AP2* mRNA nor miR172 are uniformly distributed throughout early floral primordia. Rather, *AP2* expression is initially largely restricted to future perianth and stamen primordia, whereas miR172 is specifically expressed in the center of the flower from early stages on.

Although the expression domains of miR172 and *AP2* mRNA are largely complementary, they transiently overlap, consistent with miR172 not being sufficient to clear *AP2* mRNA (Aukerman and Sakai, 2003; Chen, 2004). The stamen-to-petal conversions in *pAP3:rAP2* and to a lesser extent in *pAP3:MIM172* flowers show that miR172 regulation of *AP2* is required locally for stamen identity. Notably, mostly lateral stamens were affected in *pAP3:MIM172* flowers. As lateral stamens are initiated later than the medial ones (Smyth et al., 1990), it is possible that effects of the *pAP3:MIM172* transgene more easily overcome declining endogenous miR172 levels.

Our findings suggest that miR172 acts in a cadastral manner to prevent *AP2* activity within the outer boundaries of the C-class region. This finding has important implications for understanding how the antagonism between A and C function is implemented, which determines the boundary between perianth and reproductive organs. During early stages of flower development, miR172 and *AP2*, along with *AG*, are all expressed in stamen primordia (this work) (Drews et al., 1991). High levels of *AP2* persist in stamen primordia longer than miR172, indicating that *AP2* might be active in stamens after miR172 depletion. In severe *ap2* loss-of-function mutants, third-whorl stamens can show carpeloid characteristics (Jofuku et al., 1994), and they are reduced in number, with preferential loss of medial stamens (Bowman et al., 1991; Jofuku et al., 1994).

The classical A function is mediated by *AP1* and *AP2* and, like *AP2*, *AP1* transcripts start to be detectable in stage 1 floral primordia (Mandel et al., 1992). *AP1* is initially uniformly distributed throughout floral primordia, consistent with its early role in meristem identity, and disappears from the center of the flower in response to *AG* activation during stage 3 of flower development (Gustafson-Brown et al., 1994; Mandel et al., 1992). By contrast, *AP2* overlaps with *AG* expression in stamen primordia, confirming that *AG* does not antagonize *AP2* function at the transcriptional level.

Organs in the outer whorls of *ap2* mutants can assume reproductive organ identity, and *AG* is transcribed ectopically in the periphery of *ap2* mutant flowers (Bomblies et al., 1999; Deyholos and Sieburth, 2000; Drews et al., 1991), consistent with *AP2* repressing *AG*. However, previous evidence for the ability of ectopic *AP2* activity to repress *AG* directly has been mixed. For example, in *p35S:rAP2* plants, early *AG* expression was reported to be normal, and during later stages, *AG* was only absent from the very center of the flower (Zhao et al., 2007). However, because the 35S promoter is not always uniformly active, it is difficult to draw firm conclusions from these observations. We have directly compared *AP2* and *AG* mRNA accumulation in *pAP3:rAP2* flowers, and found that *AP2* can indeed be sufficient for local suppression of *AG* (Fig. 5).

Fig. 6. *amiR-AG*-expressing flowers. (**A**) Individual flowers of *p35S:amiR-AG-1* plants. Out of 23 T1 plants, seven had intermediate phenotypes (left), the rest had strong phenotypes (middle and right). (**B**) Individual flowers of *pAP3:amiR-AG-1* plants. Arrowheads indicate petaloid stamen, with higher magnification on the far right. Out of 23 T1 plants, eight had an intermediate phenotype (left), and five had a stronger phenotype (right). Scale bars: 2 mm.

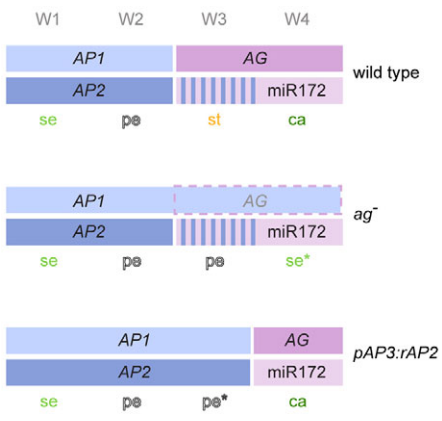

Fig. 7. Summary of interactions between A- and C-class genes. The effects on *AP1* expression are inferred from previous work (Gustafson-Brown et al., 1994; Zhao et al., 2007).

Altogether, we suggest a scenario in which miR172 is expressed in the center of floral primordia, where it acts in a cadastral manner to constrain *AP2* activity to the floral periphery (Fig. 7). *AP2* expression partially overlaps the boundary between perianth and reproductive organs in the third whorl. As a consequence, reduction of miR172 function in *pAP3:MIM172* or *pAP3:rAP2* flowers favors petal identity to be specified in organs that would normally become stamens. *AG* is apparently not sufficient to repress *AP2* expression (this work) (Zhao et al., 2007), but is itself repressed by a combination of *AP2* and other petal-specific factors (this work) (Bomblies et al., 1999; Deyholos and Sieburth, 2000; Drews et al., 1991; Krizek et al., 2006; Krizek et al., 2000).

According to the classical ABC model, the A-class function gene *AP2* specifies petal identity and therefore must be present in the center of *ag* mutant flowers (Bowman et al., 1989; Bowman et al., 1991). Paradoxically, mutant *AG* mRNA accumulates normally in the center of *ag* flowers (Gustafson-Brown et al., 1994), indicating that *AP2* is not sufficient to repress *AG* expression in its normal domain. We have shown that, although high levels of *AP2* transcript appear at least transiently in the centrally forming supernumerary floral organs in *ag* mutants, *AP2* never expands uniformly into the center of *ag* mutant flowers. Thus, petal identity might be conferred by lower levels of *AP2* than the ones required for repression of *AG*. In this scenario, low levels of *AP2* in the center of *ag* mutant flowers combined with incomplete translational repression by miR172 are sufficient to promote petal development, but do not prevent *AG* mRNA accumulation. In the periphery, by contrast, where *AP2* mRNA levels are high and miR172 is absent, *AP2* always represses *AG*. As we do not see a major effect of *AG* on *AP2* or miR172 expression, we envisage a model in which the decision whether stamens or petals develop is based on the balance between *AP2* and *AG* activities, rather than the two being mutually exclusive. Possibly related to this theme of fine-tuning *AP2* activity is the observation that *AP2* is apparently under strong negative-feedback regulation (Mlotshwa et al., 2006; Schwab et al., 2005).

A gene with an A-class function similar to that of *AP2* in *A. thaliana* has not yet been found in other species (Causier et al., 2010). Two apparent *AP2* orthologs in *Antirrhinum majus* are the functionally redundant *LIPLESS1* (*LIP1*) and *LIP2* (Keck et al., 2003). *LIP1/2* and *AP2* share similar functions in perianth organ patterning, but unlike *AP2*, *LIP1/2* activity is not required to repress the C-class gene *PLENA* (*PLE*) (Keck et al., 2003). Notably, *LIP1* expression shares features with that of *AP2*; both have been detected in emerging sepal primordia surrounding the central meristem of stage 3 and 4 flowers (this work) (Keck et al., 2003). Similar to *AP2*, *LIP1* expression declines in developing sepals. In stage 6 flowers, it is detected in the distal part of petal primordia, and, more weakly, in carpel and sometimes stamen primordia (Keck et al., 2003). The apparent *Petunia hybrida AP2* ortholog *PhAP2A* complements an *A. thaliana ap2* mutant and shares aspects of its expression pattern. Mutant analysis has, however, not revealed a function of *PhAP2A* in perianth patterning (Maes et al., 2001).

In both species, C-class gene expression is repressed by members of the miR169 microRNA family: *FISTULATA* (*FIS*) in *A. majus* and *BLIND* (*BL*) in *P. hybrida* (Cartolano et al., 2007). MiR169 targets HAP2/NF-YA transcription factors, which bind to CCAAT motifs; a pair of such conserved motifs is found in *PLE*, *AG* and its many homologs (Davies et al., 1999; Hong et al., 2003).

Outside the dicots, elegant studies have been performed on the *AP2* homolog *INDETERMINATE SPIKLET1* (*IDS1*), which is negatively regulated by miR172 and which is required to prevent the formation of extra florets in the maize inflorescence (Chuck et al., 2008; Chuck et al., 1998). Its RNA accumulates in many lateral organs, and is excluded from the center of the floral meristem soon after initiation of florets (Chuck et al., 1998), not dissimilar to the *AP2* pattern we have described.

Based on the lack of dual activities of A function genes in other species, a model proposed originally for *A. majus* (Coen and Meyerowitz, 1991) has recently been revived, in which A function is primarily required to establish floral meristem identity, which in turn leads to specification of sepal identity. In this case, only B- and C-classes of homeotic genes are required, which promote petal, stamen and carpel identity, while sepal identity results from the absence of B and C activity. By analogy, petal identity in *A. thaliana* might be either achieved by combined B and C activity (ABC model) or by B-class activity alone, as suggested in the BC

and (A)BC models (Causier et al., 2010). Perianth identity in the floral center of *ag* mutants could similarly be conferred by factors other than *AP2*, explaining the largely unaffected expression patterns of *AP2* and *AG* in *ag* mutants (this work) (Gustafson-Brown et al., 1994). Furthermore, if *AP2* activity is predominantly restricted by miR172, rather than by *AG*, both would have primarily cadastral function, with limited direct contributions to floral organ specification.

AP2 has previously been shown to affect maintenance of expression of the stem cell regulator *WUS*. In a line carrying an unusual *ap2* allele, *I28*, *WUS* expression in the shoot apical meristem is not maintained, leading to premature termination of the shoot (Würschum et al., 2006). Conversely, expression of *rAP2* from its own promoter or from the CaMV 35S promoter causes an increase in the number of floral whorls and, at least in the case of *p35S:rAP2*, this is associated with prolonged and expanded expression of *WUS* in the center of the flower (Zhao et al., 2007). We have found that region-specific overexpression of *rAP2* from the *AP3* promoter, in *pAP3:rAP2* plants, leads to ectopic formation of organs in the third and fourth whorls, apparently arising from several meristem-like centers of proliferation (Fig. 3; see Fig. S2 in the supplementary material), and this was associated with ectopic *WUS* expression (Fig. 4; see Fig. S3 in the supplementary material). Similar phenotypes are seen in plants in which *WUS* is expressed from the *AP3* promoter (Lenhard et al., 2001; Lohmann et al., 2001), but not when *AG* activity is knocked down in the same domain (Fig. 6). However, we did observe prolonged *WUS* expression in the center of the flower, suggesting the possibility that *AP2* affects *WUS* also non-autonomously. Such non-autonomous action might also be the cause of the supernumerary carpeloid organs in *pAP3:rAP2* plants, and might explain the effects of the *I28* allele of *AP2* on *WUS* expression in the vegetative shoot meristem, given that *AP2* expression is strongest in emerging leaves (Fig. 2).

In summary, we have shown that while the spatial expression patterns of *AP2* mRNA and miR172 are largely complementary, there is transient overlap in second and possibly third whorl primordia. Based on the phenotypes caused by region-specific knockdown of *AP2* and miR172, we propose that miR172 is a major factor of floral organ specification by acting in a cadastral manner to restrict AP2 activity, and thereby specifying the boundary between perianth and reproductive organs.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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