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 shoot 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 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 box 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; Wuschum 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 was 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 (Wuschum et al., 2006).

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DEVELOPMENT

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AP2 expression is regulated at the post-transcriptional level by a microRNA (miRNA), miR172 (Aukerman and Sakai, 2003; Chen, 2004, Kaschau et al., 2003; Rhodes 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, Kaschau et al., 2003; Motokiwi 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 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 Ler-1 plants were used as wild type. ag-1 (Bowman et al., 1989), ag-2 (Yanofsky et al., 1990) and dcl-11 (renamed from dcl-1-100) (Lambinger et al., 2005) 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 1:100 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 (At4g36900) 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 (At2g17930) probes were based on previously described plasmids (Leibfried et al., 2003, Yanofsky et al., 1990). The miR172 antisense LNA (locked nucleic acid, Exiqon, Vedbaek, Denmark) oligonucleotide with the sequence agctcataacgatTct (upper case, LNA, lower case: DNA) was end-labeled with the DIG 3’-End Labeling Kit (Roche) and purified with Micro 5 μm Chromatography Column (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 (Ler-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 JAF57F17 TAC (transcription-competent artificial chromosome) clone (Lin et al., 1999), which is ~32 kb in length, using a bacterial recombinase approach (Wang et al., 2003). 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/Zero (Invitrogen), respectively. The β-glucuronidase (GUS) gene was introduced into pDONR P3-P3 (Invitrogen). The three vectors were combined into a pALLIGATOR2 binary plasmid (Bestmann 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 AP2 promoter sequences were as described (Lohmann et al., 2001). The wild-type and miR172 targetting resistant (rAP2) versions of AP2 have been described (Schweb et al., 2005), and were also introduced into Gateway compatible entry plasmids. The artificial target mimicry construct MIM172

<table>
<thead>
<tr>
<th>Plasmid number</th>
<th>Description</th>
<th>Purpose</th>
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<tbody>
<tr>
<td>HW075</td>
<td>p3SS (empty)</td>
<td>Control transgenic plants</td>
</tr>
<tr>
<td>HW216</td>
<td>pAP3:MI172</td>
<td>Region-specific miR172 knock-down</td>
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<tr>
<td>HW230</td>
<td>pAP3:AP2</td>
<td>Region-specific wild-type AP2 mis-expression</td>
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<tr>
<td>HW245</td>
<td>pAP3:AP2::YFP</td>
<td>Region-specific miR172 resistant AP2 mis-expression</td>
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<tr>
<td>HW235</td>
<td>pAP3:amiR-AP2</td>
<td>Region-specific AP2 knock-down</td>
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<tr>
<td>HW210</td>
<td>p3SS:amiR-AG-1</td>
<td>Broad AG knock-down</td>
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<tr>
<td>HW209</td>
<td>p3SS:amiR-AG-1</td>
<td>Region-specific AG knock-down</td>
</tr>
<tr>
<td>HW222</td>
<td>p3SS:amiR-AG-2</td>
<td>Broad AG knock-down</td>
</tr>
<tr>
<td>HW319</td>
<td>pAP2:AP2::GUS</td>
<td>AP2 genomic reporter</td>
</tr>
<tr>
<td>JAS100</td>
<td>pAP2:AP2::YFP</td>
<td>AP2 genomic reporter</td>
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was generated by PCR-based mutagenesis of IPS1 (Franco-Zorrilla et al., 2007). Artificial miRNAs were designed using WMD (http://wmd2.weigelworld.org) (Oszerski 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 thick, 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. 1G). This last expression pattern might be related to the role of the miR172 target AP2 in integument development (León-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).

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., 2005), 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 sepal emerging on the flanks of the floral primordia (Fig. 2B,C).
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 sepal, 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 (Leon-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 Ler-1 inflorescences (Fig. 2L).

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 (Wurschum et al., 2006). We used the full-length probe to examine AP2 expression in situ hybridization in vegetative tissue. In 25-day-old, short-day grown Ler-1 accessions, 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. 2I). 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 mRNA 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 floral 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 AG 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 mRNA (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).

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. 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 (Wurschum et al., 2006; Zhao et al., 2007), we examined WUS expression by in situ hybridization in third-whorl organs enclosing the newly formed flowers. 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 tepaloid 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. S5). 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.

Evidence for AG-independent effects of AP2

Although the constitutive expression of rAP2 from the CaMV 35S promoter causes indeterminate 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 tepaloid 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. S5). 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
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 flowers show that miR172 regulation of AP2 is required locally for stamen primordia, whereas miR172 is specifically expressed in the center of the flower from early stages on.

### Specifying Floral Organ Fate

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 (Bombíles 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::tAP2 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::tAP2 flowers, and found that AP2 can indeed be sufficient for local suppression of AG (Fig. 5).
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 petal and reproductive organs in the third whorl. As a consequence, reduction of miR172 function in pAP3:MIM172 or pAP2: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) (Bombiès 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 (Miototsha 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 (Caussier 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 petal 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 (Keck et al., 2003). Similar to AP2, LIP1 expression declines in developing sepals. In stage 6 flowers, it is detectable 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 petal 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 model.
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and (A)BC models (Causier et al., 2010). Peniath 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 peniath and reproductive organs.

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Supplementary material

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