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miR172 regulates stem cell fate and defines the inner boundary of *APETALA3* and *PISTILLATA* expression domain in Arabidopsis floral meristems

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Summary

In Arabidopsis, two floral homeotic genes *APETALA2* (*AP2*) and *AGAMOUS* (*AG*) specify the identities of perianth and reproductive organs, respectively, in flower development. The two genes act antagonistically to restrict each other to their proper domains of action within the floral meristem. In addition to *AG*, which antagonizes *AP2*, miR172, a microRNA, serves as a negative regulator of *AP2*. In this study, we showed that *AG* and miR172 have distinct functions in flower development and that they largely act independently in the negative regulation of *AP2*. We uncovered functions of miR172-mediated repression of *AP2* in the regulation of floral stem cells and in the delineation of the expression domain of another class of floral homeotic genes. Given the antiquity of miR172 in land plants, our findings have implications for the recruitment of a microRNA in the building of a flower in evolution.

Keywords

miR172; flower development; stem cells; B function; *APETALA2*; *AGAMOUS*

Introduction

The shoot apical meristem (SAM) formed during embryo-genesis is the ultimate source of all above-ground structures of the plant. The dynamic balance between differentiation and stem cell renewal in the SAM is achieved through a negative feedback loop involving the *CLAVATA* (*CLV*)*-1*, *-2* and *-3* genes, and a homeodomain transcription factor, WUSCHEL (WUS) (reviewed in Brand *et al*., 2001; Clark, 2001; Sharma *et al*., 2003). *CLV3* encodes a small, secreted protein, presumably a ligand for the potential transmembrane receptors CLV1 and CLV2. *WUS* RNA is found in a small number of cells underneath the stem cells. While *WUS* specifies the overlying cells as stem cells, *CLV*-mediated signaling from the stem cells in turn restricts the *WUS* expression domain. APETALA2 (AP2), a transcription factor known to act in floral patterning and seed development (Jofuku *et al*., 1994), regulates stem cell maintenance in the SAM through the *CLV–WUS* pathway (Würschum et al., 2006).

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The *CLV–WUS* pathway also functions in the floral meristem to regulate floral stem cells (reviewed in Sharma *et al*., 2003). Unlike the SAM, however, the floral meristem terminates upon production of the final whorl of organs, carpels. The timely termination of the floral meristem requires AGAMOUS (AG), a MADS-domain transcription factor (Lenhard *et al*., 2001; Lohmann *et al*., 2001; Yanofsky *et al*., 1990). In severe *ag* mutants, the floral meristem continues to put out floral organs to produce a flowers-within-flower phenotype (Bowman *et al*., 1989).

In Arabidopsis flowers, two types of perianth organs, sepals and petals, are found in the outer two whorls and the reproductive organs, stamens and carpels, are found in the inner two whorls. The identities of the four types of floral organs are specified by three classes of regulatory genes, the A, B and C genes (reviewed in Jack, 2004; Krizek and Fletcher, 2005). The class A genes, *APETALA1* (*AP1*) and *AP2*, specify sepal identity in whorl 1 and petal identity in whorl 2 together with the class B genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*). The B genes specify stamen identity in whorl 3 together with the C gene *AG*. *AG* determines carpel identity in whorl 4. Consistent with the spatially restricted activities of the ABC genes in the floral meristem, transcripts of the ABC genes, with the exception of *AP2*, are found in two adjacent whorls. Studies on flower development in other plant species suggest that the role of A function in specifying perianth identity is restricted to Brassicaceae (reviewed in Litt, 2007). Even in Brassicaceae, it is possible that the sole role of the A function is to specify floral meristem identity, and perianth organs (such as sepals) represent the ground state of floral organs (reviewed in Litt, 2007). Whether *AP2* plays a direct or indirect role in specification of perianth identity does not influence the interpretations of our studies in this paper.

Antagonism between *AP2* and *AG* is crucial in floral patterning. Loss-of-function *ap2* alleles have reproductive organs in place of perianth organs, indicating the expansion of *AG* activity into the outer two whorls (Bowman *et al*., 1991b). Loss-of-function *ag* alleles have petals in place of stamens and a new flower in place of carpels (Bowman *et al*., 1991b). The replacement of all reproductive organs by perianth organs suggests that *AP2* is active throughout the flower in *ag* mutants. At the molecular level, *AP2* restricts *AG* activity to the inner two whorls by preventing *AG* transcription in the outer two whorls (Drews *et al*., 1991; Sieburth and Meyerowitz, 1997). *AG*, however, does not repress *AP2* expression and *AP2* RNA is found throughout the flower (Jofuku *et al*., 1994). It is unknown how *AG* counteracts *AP2* activity in the inner two whorls. Ectopic *AG* expression in the outer two whorls results in phenotypes resembling those of *ap2* mutants, indicating that ectopic *AG* is able to counteract *AP2* activity in the outer two whorls (Mizukami and Ma, 1992).

In recent years, a microRNA, miR172, has been demonstrated to be another negative regulator of *AP2* (Aukerman and Sakai, 2003; Chen, 2004). miR172 is initially present throughout the floral meristem but is concentrated in the inner two whorls after floral stage 7 (Chen, 2004). Misexpression of *MIR172* genes with the 35S promoter results in flowers resembling *ap2* mutants (Aukerman and Sakai, 2003; Chen, 2004). Expression of a miR172-resistant version of *AP2* cDNA, *AP2m3*, but not wild-type *AP2* cDNA, leads to dramatic floral phenotypes, indicating that miR172 acts in the flower to repress *AP2* (Chen, 2004). Aspects of the *35S::AP2m3* phenotypes are similar to those of *ag* mutants (Bowman *et al*., 1991b), such as the loss of floral determinacy and the transformation of reproductive organs into perianth organs. This raises the possibility that miR172 mediates the negative regulation of *AP2* by *AG*. However, these data do not preclude miR172 and *AG* acting as independent negative regulators of *AP2* in flower development.

In this study, we evaluated the role of miR172-mediated regulation of *AP2* in flower development by analyzing the effects of expressing miR172-resistant *AP2* under its own promoter. We found that this regulation is crucial for floral patterning in the inner two whorls.

We uncovered functions of miR172, and thereby probably of *AP2*, in the control of floral stem cell fate and in the delineation of the inner boundary of the B gene expression domain. Furthermore, we showed that miR172 and *AG* have distinct roles in flower development and act independently as negative regulators of *AP2*.

Results

Effects of impaired AP2 regulation by miR172 on flower development

Previous studies demonstrated that miR172 is a negative regulator of *AP2* in the flower but failed to delineate the role of this regulation in flower development (Aukerman and Sakai, 2003; Chen, 2004). Expression of *AP2m3* with the strong 35S promoter led to two major types of floral defect (Figure 1a–c; Chen, 2004). Type I flowers had numerous petals surrounding an indeterminate floral meristem (Figure 1b) while type II flowers had numerous stamens flanking an indeterminate floral meristem (Figure 1c). It was unclear which phenotype, if any, reflected the outcome of loss of miR172 function because the strong 35S promoter may have contributed to aspects of the phenotypes. miR172 is encoded by at least five genes in the Arabidopsis genome. A *mir172a-1 mir172a-2* double mutant does not exhibit any floral defects (LZ and XC, unpubl. result). A quintuple mutant in the *MIR172* genes may be necessary to evaluate the developmental role of miR172. In the absence of such a mutant, we sought to evaluate the function of miR172-mediated regulation of *AP2* in flower development by analyzing lines that express *AP2m3* under the control of the *AP2* regulatory sequences including the promoter, 5′ untranslated region (UTR) and 3′ UTR.

We cloned a 5.4-kb fragment upstream of the *AP2* cDNA as the *AP2* promoter. This fragment encompassed the upstream sequence previously used in an *AP2* clone to rescue *ap2* mutant phenotypes (Jofuku *et al*., 1994). When fused to the GUS reporter and introduced into wild-type plants, this sequence led to ubiquitous GUS expression in the flower (LZ and XC, unpubl. result) in agreement with the ubiquitous presence of *AP2* RNA detected by *in situ* hybridization (Jofuku *et al*., 1994). We fused this fragment to the full-length wild-type (*AP2WT*) or *AP2m3* cDNAs and introduced the *pap2::AP2WT* or *pAP2::AP2m3* constructs into wild-type Arabidopsis plants. None of the *pAP2::AP2m3* lines had flowers with the type I *35S::AP2m3* phenotypes, suggesting that the type I phenotypes were partly because of the 35S promoter. Over 30% of the independent T_1 *pAP2::AP2m3* transgenic lines, but none of the *pAP2::AP2WT* lines, showed floral defects similar to the type II *35S::AP2m3* lines. In brief, the outer two floral whorls were normal in organ identity, but numerous stamens were found internal to the petals (Figure 1d). Scanning electron microscopy showed that the stamen primordia arose in a spiral, rather than whorled, phyllotaxy (Figure 1e). In some lines, large numbers of unfused carpels were found internal to the numerous stamens, or occasional unfused carpels were found among the numerous stamens (Figure 1f). Similar phenotypes were also present in some *35S::AP2m3* type II lines.

As *pAP2::AP2m3* transgenic lines mainly displayed type II *35S::AP2m3* phenotypes, we conclude that the type II phenotype is a better measure of the consequence of impaired miR172-mediated repression of *AP2* in flower development. To our knowledge, phenotypes similar to those of *pAP2::AP2m3* flowers have not been found in known floral mutants, suggesting that miR172-mediated regulation of *AP2* performs a previously unknown function in flower development. In fact, the presence of stamens and sometimes carpels in *pAP2::AP2m3* flowers suggests that miR172, in contrast to *AG*, does not play a major role in the specification of reproductive organ identities.

In order to further characterize the novel phenotypes of *pAP2::AP2m3* flowers to better understand the role of miR172 in flower development, it was necessary to compare the effects of the transgene in various known floral mutant backgrounds. This required crossing the

transgene from an established *pAP2::AP2m3* line into various mutants so that transgene location and number would remain the same across different genotypes to warrant phenotypic comparison. However, it was not possible to do so for *pAP2::AP2m3* lines as they were male and female sterile. Although numerous stamens were present, they seldom bore pollen. In contrast, type II *35S::AP2m3* lines, which were almost identical to *pAP2::AP2m3* in floral patterning defects, were fully male fertile (but female sterile). The difference was probably because of the 35S promoter not being as active as the *AP2* promoter in the pollen. As we were only focusing on early patterning roles of miR172, type II *35S::AP2m3* lines were an excellent genetic resource to represent impaired *AP2* regulation by miR172. We first established several type II *35S::AP2m3* lines in which the transgene was in a single locus. The lines were maintained by crossing *35S::AP2m3* to wild type such that the transgene was always in a hemizygous configuration. The transgene was introduced into other genetic backgrounds by two consecutive crosses into various recessive mutants such that the transgene was hemizygous in homozygous mutant backgrounds.

To confirm that the phenotypes observed for *35S::AP2m3* in various floral mutant backgrounds truly reflected those of *pAP2::AP2m3* in the corresponding genetic backgrounds, we also transformed various floral mutants with the *pAP2::AP2m3* construct and examined multiple T_1 transgenic lines. *pAP2::AP2m3* was introduced into *wus-1*, *lfy-6*, *ufo-2*, *ap3-3*, *pi-1*, and *clv3-1*. The phenotypes of *pAP2::AP2m3* in these mutant backgrounds (Supplementary Figure S1) were nearly identical to those of *35S::AP2m3* in the corresponding mutant backgrounds. Our analyses as described below were conducted largely with the *35S::AP2m3* lines. From this point on, we simply refer to the established type II *35S::AP2m3* lines as *35S::AP2m3*, which we use to represent impaired miR172-mediated regulation of *AP2*.

Role of miR172 in the regulation of floral stem cells

At least two parallel genetic pathways regulate floral stem cells through *WUS*. *CLV* signaling restricts the number of *WUS*-expressing cells. Loss-of-function mutations in *CLV* genes result in an enlarged *WUS* expression domain in the floral meristem and an enlarged floral meristem that produces a larger number of floral organs (Schoof *et al*., 2000). *AG*, however, does not affect the domain of *WUS* expression but shuts down *WUS* expression at around stage 7 when carpel primordia are formed (Lenhard *et al*., 2001; Lohmann *et al*., 2001). The *CLV* and *AG* pathways probably act in parallel as the *clv1 ag* double mutant has greatly enlarged and often fasciated floral meristems, a phenotype that is more severe than that of either single mutant (Clark *et al*., 1993).

The fact that *35S::AP2m3* flowers had numerous stamens flanking an indeterminate floral meristem suggested that miR172-mediated repression of *AP2* played a role in the control of floral stem cells. Introducing the *wus-1* mutation into the *35S::AP2m3* background completely abolished the indeterminate phenotype (Figure 2a,b). In fact, *35S::AP2m3 wus-1* flowers were identical to *wus-1* flowers in that the floral meristem terminated in a single stamen. The complete epistasis of *wus-1* to *35S::AP2m3* indicated that the effect of the transgene is mediated by *WUS*. A probable underlying mechanism is that de-repressed *AP2* expression in *35S::AP2m3* results in reduced *AG* expression and secondarily to prolonged *WUS* expression. Indeed, the onset and the initial domain of *WUS* expression in *35S::AP2m3* flowers were similar to wild type (Figure 3a,b), but *WUS* remained expressed in *35S::AP2m3* throughout flower development as in *ag* mutants (Figure 3c–e). As in wild type, *AG* RNA was found in the center of stages 3–4 *35S::AP2m3* floral meristems (Figure 3j and data not shown; Drews *et al*., 1991). *AG* RNA continues to be present in the inner two whorls throughout flower development in wild type (Figure 3j; Bowman *et al*., 1991a). In *35S::AP3m3* flowers, however, *AG* RNA was much reduced, but apparently not totally abolished, at the very apical end of the meristem

starting from stage 6 (Figure 3k,l). These observations were consistent with *AP2m3* promoting stem cell fate, at least in part, through the repression of *AG*.

Our genetic evidence indicated that *AP2m3* also acted independently of *AG* in the regulation of floral stem cells. In the *ag-1* background, *35S::AP2m3* still led to a dramatic increase in floral organ number (Figure 2d,e) and floral meristem size (compare Figure 3c with 4e), indicating that *AP2m3* exerted a large effect on floral stem cells through an *AG*-independent, perhaps the *CLV*, pathway. In fact, *35S::AP2m3* and *clv* mutants exhibited a number of similarities. Firstly, *35S::AP2m3* floral meristems were taller than wild type starting at stage 3 (Figure 3g,h), a phenotype similar to *clv* mutants (Clark *et al*., 1993). Although the initial domain of *WUS* expression was normal in young *35S::AP2m3* flowers (Figure 3b), an obvious expansion of the *WUS* expression domain was often observed in later flowers, and very late stage flowers showed an expansion of *WUS* expression to the entire meristem and to young organ primordia (Figure 3f). Secondly, *35S::AP2m3* and *clv1-4* flowers showed near identical patterns of *AG* and *AP1* expression. *AG* RNA was reduced in the center of the meristem in *35S::AP2m3* flowers (Figure 3k,l) as well as in *clv1-4* flowers (Clark *et al*., 1993). While *AP1* RNA is normally restricted to the outer two floral whorls after stage 3 in wild-type flowers (Figure 3g; Mandel *et al*., 1992), it was found in the center of the floral meristems in *35S::AP2m3* (Figure 3h,i) and in *clv1-4* (Clark *et al*., 1993) plants. Finally, introduction of *ag-1* into *35S::AP2m3* resulted in a synergistic interaction such that the floral meristem was greatly expanded (compare Figure 4d,e), which was similar to the effects of combining *ag* and *clv* mutations (Clark *et al*., 1993). These observations support but do not prove that miR172-mediated repression of *AP2* regulates floral stem cells through the *CLV* pathway. As *AP2* was shown to regulate stem cell maintenance in the SAM through the *CLV* pathway (Würschum *et al*., 2006), it is likely that *AP2* has a similar function in floral meristems.

Assuming that *35S::AP2m3* acts in the *CLV* pathway, we tested whether it regulated floral stem cells exclusively through the *CLV* pathway. If this were the case, we would expect the phenotypes of *35S::AP2m3* flowers to be identical to those of *35S::AP2m3 clv3-1* flowers. We generated transformants of *pAP2::AP2m3* in the *clv3-1* background. Flowers of T_1 *pAP2::AP2m3 clv3-1* plants differed from *pAP2::AP2m3* flowers in that they accumulated a mass of apparently undifferentiated tissue in the center (Figure 2c). The fact that *pAP2::AP2m3 clv3-1* flowers have more severe phenotypes than either *pAP2::AP2m3* or *clv3-1* flowers indicates that de-repressed *AP2* does not act exclusively through the *CLV* pathway. In summary, our analysis shows that miR172 is a crucial factor in the regulation of floral stem cells and it acts through an *AG*-dependent pathway and an *AG*-independent, perhaps *CLV*, pathway and that these two pathways converge on *WUS*.

35S::APm3 leads to an expansion of B gene expression domain

We examined the unexpected role of miR172-mediated repression of *AP2* on stamen number in flower development. Specification of stamen identity requires *AP3* and *PI*. The presence of numerous stamens in *35S::AP2m3* suggests that *AP3* and *PI* are expressed in a larger domain covering all the stamen primordia. Introduction of either *ap3-3* or *pi-1* loss-of-function mutations into *35S::AP2m3* transformed the stamens to sepal-like organs or filaments (Figure 2f and Supplementary Figure S1e), indicating that *AP3* and *PI* were active in a domain containing all the stamens in *35S::AP2m3* flowers. We examined the accumulation of *AP3* and *PI*RNAs in developing flowers by *in situ* hybridization. In stages 3–5 *35S::AP2m3* flowers, *AP3* RNA appeared to occupy whorls 2 and 3 as in wild type (data not shown and Figure 4a; Jack *et al*., 1992). In later *35S::AP2m3* flowers, *AP3* RNA continued to be present in all internal stamen primordia (Figure 4d). In wild-type flowers, *PI*RNA is initially present in whorls 2–4 but the whorl 4 expression soon abates and a whorl 2–3 pattern is established and maintained throughout flower development (Figure 4f; Goto and Meyerowitz, 1994). In *35S::AP2m3*

flowers, the initiation of *PI* expression appeared normal, but *PI* RNA never disappeared from the center of the meristem (Figure 4i). In addition, *PI* RNA was present in all stamen primordia (Figure 4i). These data suggested that de-repressed *AP2* expression led to an expansion of the *AP3/PI* expression domain towards the center of the meristem.

Floral meristem identity genes *LEAFY (LFY)* and *UNUSUAL FLORAL ORGANS* (*UFO*) are required for the initiation of B gene expression (Levin and Meyerowitz, 1995; Parcy *et al*., 1998). We asked whether the expansion of the B gene expression domain caused by *35S::AP2m3* requires the activity of the floral meristem identity genes. Severe mutant alleles in *LFY* and *UFO* were introduced into *35S::AP2m3*. *35S::AP2m3 lfy-6* and *35S::AP2m3 ufo-2* flowers had sepal-like organs or filaments rather than stamens (Supplementary Figure S1f,g), suggesting that *LFY* and *UFO* are necessary for B gene activation by *35S::AP2m3*. Indeed, *in situ* hybridization showed that *AP3* and *PI* signals were patchy and reduced in intensity in *35S::AP2m3 lfy-6* and *35S::AP2m3 ufo-2* flowers (Supplementary Figure S1h,i and data not shown). Therefore, de-repressed *AP2* expression was not sufficient to induce *AP3* and *PI* expression. Our data are more consistent with a function of *35S::AP2m3* in affecting the inner boundary of the B gene expression domain rather than activating B gene expression.

Mutations in the *SUPERMAN* (*SUP*) gene lead to an increased number of stamens (Bowman *et al*., 1992). It is possible that the increase in stamen number in *35S::AP2m3* was caused by reduced *SUP* expression. However, this was unlikely because the *35S::AP2m3* phenotype is much more severe than that of *sup* loss-of-function alleles, which show a modest number of extra stamens (Bowman *et al*., 1992). Indeed, *SUP* expression was not affected in *35S::AP2m3* (Figure 5b).

AP2 and AG control B gene expression domain in the floral meristem

We asked whether a potential role for *AP2* in the control of the B gene expression domain was supported by loss-of-function *ap2* mutant defects. Although a role for *AP2* in the control of the B gene expression domain had not been proposed, earlier observations (Jack *et al*., 1992) were consistent with such a role. Jack *et al*. found that *AP3* RNA was at a much lower level and occupied a smaller area in *ap2-2* flowers compared with wild type. Furthermore, they reported that *AP3* expression was restored by introducing an *ag* mutation into *AP2-2*. We confirmed these observations by comparing the intensity and the domain of *AP3* RNA among wild-type, *ap2-2*, and *ap2-2 ag-1* flowers on the same glass slide (Figure 4a–c). In addition, we found that *PI* expression was drastically reduced in *ap2-2* (Figure 4f,g) and restored in *ap2-2 ag-1* (Figure 4h). Therefore, *AP2* promotes the levels of *AP3* and *PI* expression and establishes the size of the expression domain by counteracting *AG*. The antagonistic interaction between *AP2* and *AG* defines the domain of B function. By repressing *AP2* in the inner two whorls, miR172 plays a key role in defining the inner boundary of the B gene expression domain.

The *AP2-AG* antagonistic interaction appeared to affect *AP3* and *PI* expression somewhat differently. While *PI* RNA was present in the center of the meristem of *35S::AP2m3* flowers (Figure 4i), *AP3* RNA was not (Figure 4d). In fact, *AP3* RNA was also absent from the center of the meristem in *ag-1* (Figure 4c) or *35S::AP2m3 ag-1* flowers (Figure 4e). This indicates that a gene(s) other than *AP2*, *AG* or *MIR*172 causes the absence of *AP3* expression from the center of the meristem.

miR172 and AG act independently on AP2 in flower development

As negative regulators of *ap2* in flower development, miR172 and *AG* may act in the same genetic pathway or in parallel genetic pathways. We evaluated the relationship between *AG*

and miR172 in the negative regulation of *AP2*. We asked whether one is functional in the absence of the other. Introduction of *ag-1* into *35S::AP2m3*, whose pheno-type represented impaired *AP2* regulation by miR172, resulted in the transformation of stamens into petals (Figure 2a,e), indicating that *AG* was still active in antagonizing *AP2* in the absence of miR172. Conversely, introducing *35S::AP2m3* into *ag-1* resulted in a great increase in organ number (Figure 2d,e) and floral meristem size (Figure 3c and 4e), indicating that miR172 was still repressing *AP2* in an *ag* mutant. Consistent with these genetic observations, *AG* RNA was still present in *35S::AP2m3* flowers (Figure 3k,l) and miR172 was still present in *ag-1* inflorescences (Figure 5a). Therefore, miR172 and *AG* act independently on *AP2* in flower development. The drastic floral defects of either *35S::AP2m3* or *ag-1* flowers underscore the crucial function of both miR172 and *AG* as negative regulators of *AP2*. Previous experiments to mis-express *AG* with the 35S promoter led to the conclusion that *AG* is sufficient to counteract *AP2* when ectopically present in the outer two whorls. One might extrapolate to assume that *AG* is normally sufficient to counteract *AP2* in the inner two whorls. However, our data show that *AG* is not sufficient to counteract *AP2* in the inner two whorls. Without miR172, elevated *AP2* levels led to severe consequences for flower development.

Discussion

miR172-mediated repression of AP2 is crucial for the patterning of the inner two whorls

Our detailed characterization of the *pAP2::AP2m3* and type II *35S::AP2m3* phenotypes led to the following conclusions concerning the role of miR172-mediated repression of *AP2* in floral patterning (Figure 6). Firstly, miR172-mediated repression of *AP2* is crucial for maintaining floral meristem size and for the timely termination of floral stem cells. The role of miR172 on floral meristems is achieved through both an *AG*-dependent and an *AG*-independent pathway. Secondly, miR172-mediated repression of *AP2* defines the inner boundary of the domain of B gene expression. This role of miR172 is probably achieved through an *AG*-dependent pathway. Thirdly, miR172-mediated repression of *AP2* plays little role in the specification of reproductive organ identities as does *AG*,as *35S::AP2m3* flowers still have stamens and sometimes carpels. It appears that de-repressed *AP2* expression has the most adverse impact on floral patterning in the center of the floral meristem (which would become the fourth whorl in wild-type flowers) such that the meristem continues to proliferate and that the B gene expression domain expands into the center. De-repressed *AP2* expression does not affect the identity of the third whorl organs (stamens). Many of the effects on floral patterning by de-repressed *AP2* expression, such as prolonged *WUS* expression and expansion of B gene expression towards the center of the meristem, can be explained by compromised *AG* expression in the fourth whorl of the flower. However, de-repressed *AP2* expression affects floral meristem size independently of *AG*.

Role of AP2 in flower development

It is necessary to consider to what extent the consequences of de-repressed *AP2* in floral patterning reflect the normal role of *AP2*. While de-repressed *AP2* expression leads to an expansion of the B gene domain, prolonged *WUS* expression, and increased meristem size, does *AP2* normally regulate the B gene expression domain, timing of *WUS* expression, and floral meristem size? It is possible that *AP2* does not normally act in these processes, but elevated levels of *AP2* allow it to feed into other pathways that do. The key to addressing the question lies in whether *ap2* loss-of-function mutations affect these developmental processes. Evidence in the literature (Jack *et al*., 1992) and from this study supports the conclusion that *AP2* promotes the B gene expression domain by antagonizing *AG*. Our preliminary studies on *WUS* expression in *ap2-2* mutant flowers did not reveal any difference from wild type (LZ and XC, unpubl. results). Therefore, at present, we do not have evidence from *ap2* loss-of-function alleles that *AP2* regulates the timing of *WUS* expression. We have not compared the sizes of

ap2 mutant and wild-type floral meristems and thus cannot conclude on whether *AP2* plays a role in the regulation of floral meristem size. However, *AP2* has been shown to play a role in stem cell maintenance in the SAM through the *CLV* pathway (Würschum *et al*., 2006). It is possible that *AP2* has a similar function in the control of floral stem cells.

miR172 and AG have distinct roles in floral patterning

What is the relationship between *AG* and miR172 in floral patterning? *AG* has long been known to counteract *AP2* activity in the inner two whorls, but the underlying mechanism is unknown. Accumulation of *AP2* RNA is ubiquitous in the four floral whorls and is similar in wild type and *ag* mutants (Jofuku *et al*., 1994), suggesting that *AG* does not repress *AP2* at the transcriptional level. miR172 was found to serve as a translational repressor of *AP2* mRNA (Aukerman and Sakai, 2003; Chen, 2004). One attractive hypothesis is that *AG* regulates the transcription of *MIR172* genes and miR172 leads to reduced *AP2* protein levels in the inner two whorls. Our studies show that this model is incorrect. Firstly, miR172 levels in wild type and *ag-1* flowers are similar, indicating that *AG* is not required to turn on *MIR172* genes. Secondly, the fact that *35S::AP2m3 ag-1* flowers are phenotypically different from either *35S::AP2m3* or *ag-1* flowers demonstrates that miR172 is active in *ag-1* and that *AG* is active in *35S::AP2m3*. Therefore, miR172 and *AG* act independently in floral patterning.

miR172 has an ancient origin in land plants (Axtell and Bartel, 2005) and must therefore have been recruited by angiosperms to build the flower. Angiosperm flowers display the basic types of floral organs but vary dramatically in the number of the organs among species. While some species have a small number of stamens in the flower, others, such as representatives of the Magnoliaceae, have numerous stamens arranged in a spiral phyllotaxy as seen in *35S::AP2m3* flowers. One cannot help but wonder whether miR172-mediated repression of *AP2* plays a role in the evolution of floral morphology.

Experimental procedures

Constructs and transgenic lines

For the generation of *pAP2::AP2m3* and *pAP2::AP2WT* lines, an *AP2* promoter was amplified by PCR from Col genomic DNA with the primers AP2p41 (5′-AAG CTG CAG TTA GGC CCG ACC TAT CGT CCA TC-3′) and AP2p42 (5′-AAA CTG CAG CTA AAG AGA GAG AGA GAA GAA AAT AAA ATA-3′). The PCR product was digested by *PstI* and ligated into the pPZP211 vector to generate pPZP211-pAP2. The full-length *AP2WT* and *AP2m3*cDNAs were obtained by digestion of the plasmids pBSSK-AP2WT and pBSSK-AP2m3 (Chen, 2004) with *SacI* and were ligated downstream of the *AP2* promoter in pPZP211-pAP2. The resulting constructs *pAP2::AP2WT* and *pAP2::AP2m3* were delivered into wild-type Arabidopsis plants of the Landsberg *erecta (Ler)* ecotype by agroinfiltration.

pAP2::AP2m3 was also transformed into *clv3-1, ufo-2, lfy-6, wus-1, ap3-3,* and *pi-1* mutant backgrounds. For *clv3-1* and *ufo-2*, homozygous plants were used for transformation. For *lfy-6, wus-1, ap3-3,* and *pi-1*, populations that segregated each of these mutations were used for transformation.

Introduction of 35S::AP2m3 into various mutant backgrounds

35S::AP2m3 lines in which the transgene was inserted into a single locus were first obtained by crossing 18 independent T_1 lines with type II phenotypes as the pollen donor with *Ler* and following the segregation of the phenotypes in the F_1 populations. Ones with a 1:1 segregation of *35S::AP2m3* versus wild-type phenotypes were retained. Because of the female sterility of the lines, they were maintained in the form of F_1 seeds of crosses to *Ler*.

The *35S::AP2m3* transgene from the single-locus lines was introduced into *ap3-3*, *pi-1*, *ag-1*, *wus-1*, *ufo-2*, and *lfy-6* backgrounds by two consecutive crosses with the homozygous mutants (in the case of *ap3-3*, *pi-1*, *ufo-2*, and *lfy-6*) or heterozygous mutants when the homozygous mutants were female sterile (in the case of *ag-1* and *wus-1*). With the homozygous mutants, a cross was first performed with an *35S::AP2m3* line and F_1 plants exhibiting *35S::AP2m3* phenotypes (they would be hemizygous for the *35S::AP2m3* transgene and heterozygous for the mutation) were crossed to the corresponding homozygous mutants again. F_1 plants from the second crosses were screened to identify the homozygous mutants containing the transgene. In the crosses to *ag-1/+* and *wus-1/+*, F_1 plants with *35S::AP2m3* phenotypes were first genotyped to identify ones that were also *ag-1/+* or *wus-1/+*. These plants were then crossed again with *ag-1/+* or *wus-1/+* plants. *35S::AP2m3* in *ap3-3*, *pi-1*, *ufo-2*, *ag-1* and *lfy-6* backgrounds was recognized by novel phenotypes distinguishable from both parents. The *35S::AP2m3* crosses to *wus-1/+* did not give any phenotypes other than those of *35S::AP2m3* or *wus-1*. *35S::AP2m3 wus-1* plants were identified by PCR genotyping plants with *wus-1* phenotypes for the presence of *35S::AP2m3*.

In situ hybridization, RNA filter hybridization, and RT-PCR

In situ hybridization was carried out as described (Chen *et al*., 2002) and RNA filter hybridization to detect miR172 was performed as described (Park *et al*., 2002). For RT-PCR, total RNA was first isolated from wild-type and *35S::AP2m3* inflorescences as described (Chen *et al*., 2002). 2.5 μg of total RNA from each genotype was treated with DNaseI and subjected to reverse transcription. A portion of the reverse transcription reaction was amplified by PCR with *SUP* (SUP-F: 5′-CTT GGA GCT TGA GAT TGG-3′ and SUP-R: 5′-CGG TAA CAA GCG CAT ACA-3′) and *UBQ* (NUBQ: 5′-GGT GCT AAG AAG AGG AAG AAT-3′ and CUBQ: 5′-CTC CTT CTT TCT GGT AAA CGT-3′) primers. Twenty, 25, and 30 cycles of PCR were performed for *UBQ* and 30, 35, and 40 cycles of PCR were performed for *SUP* to ensure that the reactions did not reach saturation at the number of cycles presented in Figure 5.

Scanning electron microscopy

Scanning electron microscopy was performed with fresh flowers using a Hitachi TM1000 scanning electron microscope (http://www.hitachi.com/).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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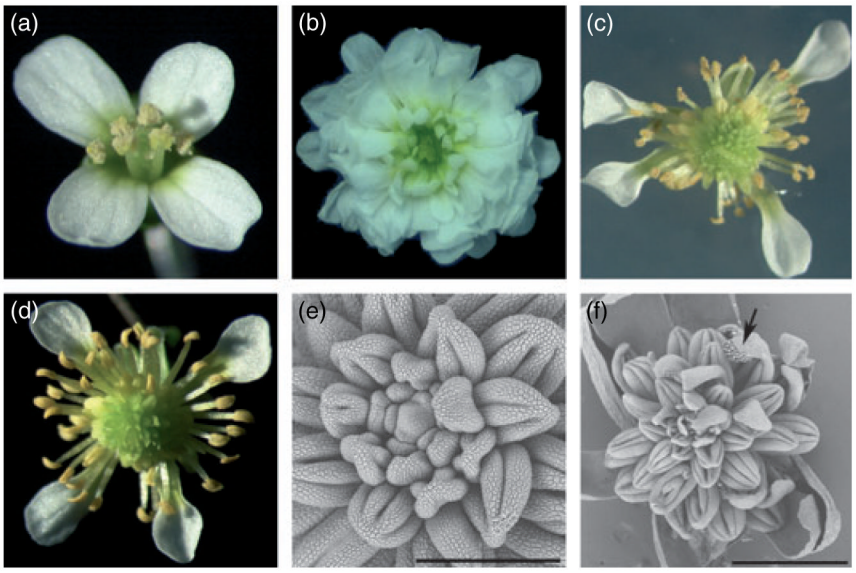

Figure 1.
Floral phenotypes of *35S::AP2m3* and *pAP2::AP2m3* transgenic lines.
(a) Wild type.
(b) *35S::AP2m3*, type I.
(c) *35S::AP2m3*, type II.
(d) *pAP2::AP2m3*.
(e, f) Scanning electron micrographs of *pAP2::AP2m3* flowers. (e) A close-up of the center of the flower showing the indeterminate floral meristem and the spiral arrangement of the emerging stamen primordia. (f) Occasional carpelloid organs with stigmatic papillae (arrow) are found in *pAP2::AP2m3* flowers. Scale bars: 300 μm in (e) and 1 mm in (f).

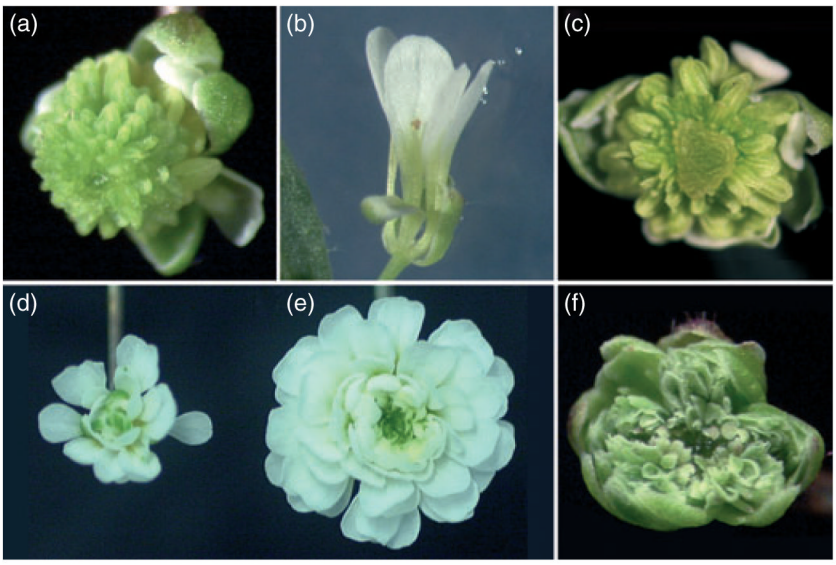

Figure 2.

35S::AP2m3 or *pAP2::AP2m3* in various genetic backgrounds. In all cases, the trans-gene is hemizygous and is in a homozygous mutant background.

(a) The *35S::AP2m3* line that was used to cross with various mutants. Its phenotype serves as a reference for comparison with that of *35S::AP2m3* in various genetic backgrounds.

(b) *35S::AP2m3 wus-1*.

(c) A flower from a *pAP2::AP2m3 clv3-1* T1 transgenic plant. Note the mass of apparently undifferentiated cells in the center.

(d, e) An *ag-1* flower (d) and a *35S::AP2m3 ag-1* flower (e) at the same magnification.

(f) *35S::AP2m3 pi-1*.

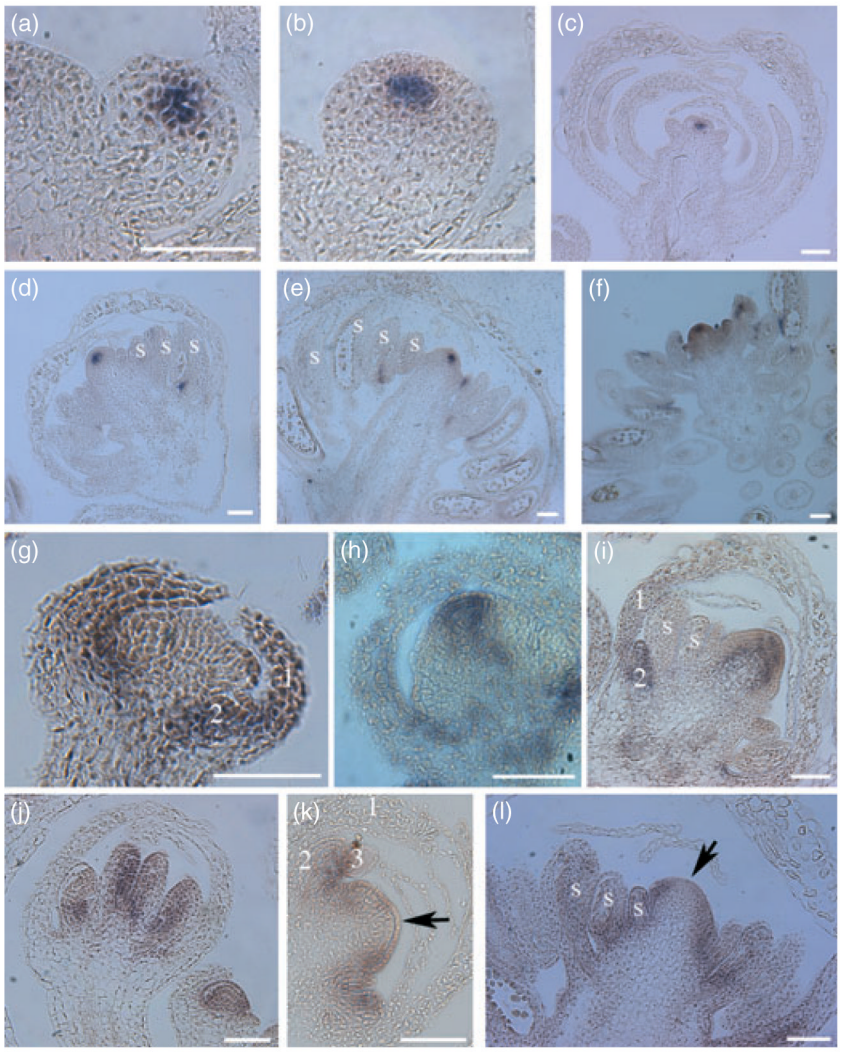

Figure 3.

In situ hybridization for the detection of *WUS*, *AP1*, and *AG* RNAs in flowers of various genotypes.

(a–f) Detection of *WUS* RNA. (a) A wild-type stage 2 flower. (b) A *35S::AP2m3* stage 2 flower. (c) An old *ag-1* flower. (d–f) *35S::AP2m3* flowers of increasingly advanced stages in development. (g–i) Detection of *AP1* RNA. (g) A wild-type stage 6 flower. (h,i) *35S::AP2m3* stage 6 (h) and older (i) flowers.

(j–l) Detection of *AG* RNA. (j) wild-type stage 8 (left) and stage 4 (right) flowers. (k,l) *35S::AP2m3* stage 6 (k) and older (l) flowers. The arrows mark the center of the meristems where *AG* RNA levels are low compared with the surrounding areas. The numbers indicate the floral whorls. ‘s’ represents stamens or stamen primordia. Scale bars: 50 μm.

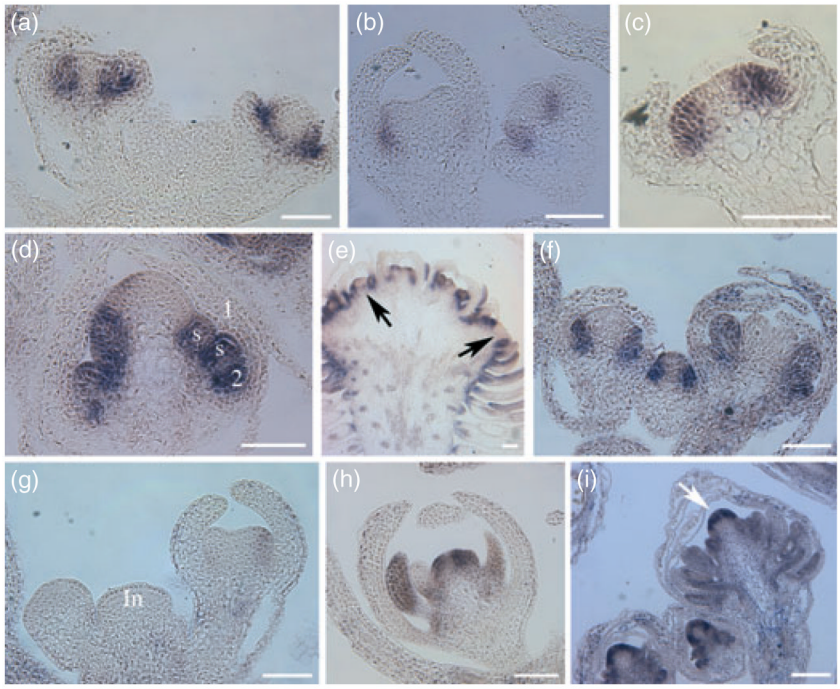

Figure 4.

In situ hybridization for the detection of *AP3* and *PI* RNAs in flowers of various geno-types.

(a–e) *AP3 in situ* hybridization.

(a) Stage 6 (left) and stage 4 (right) wild-type flowers.

(b) Stage 6 (left) and stage 4 (right) *ap2-2* flowers.

(c) A stage 4-5 *ap2-2 ag-1* flower.

(d) A stage 6 *35S::AP2m3* flower.

(e) An old *35S::AP2m3 ag-1* flower in which the meristem has fasciated and has been subdivided into many second-order floral meristems. The arrows indicate that the center of the second-order meristems has little *AP3* RNA.

(f–i) *PI in situ* hybridization. (f) Three wild-type flowers of various stages with strong *PI* expression in the second and third whorls. (g) A longitudinal section through the inflorescence (In) of *ap2-2*. *PI* expression is barely detectable in the stage 2 (left) and stage 6 (right) flowers. (h) An *ap2-2 ag-1* flower showing *PI* expression. (i) Three *35S::AP2m3* flowers of various stages showing strong and ectopic (arrows) *PI* expression. The numbers indicate the floral whorls. ‘s’ represents stamen primordia. Scale bars: 50 μm.

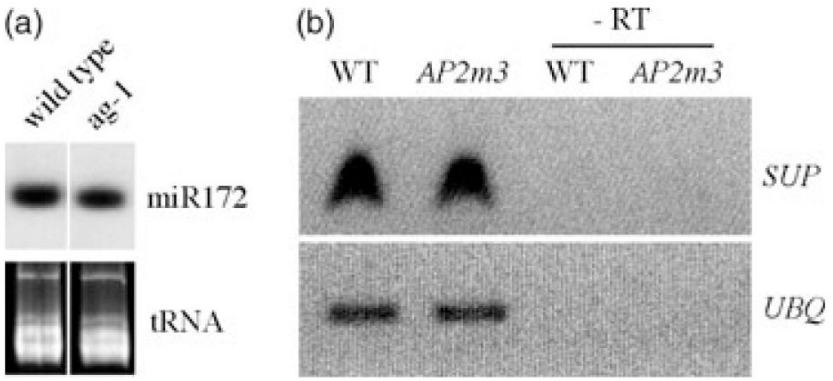

Figure 5.

miR172 and *SUP* mRNA levels in various genotypes.

(a) miR172 levels in inflorescences of wild-type and *ag-1* plants as determined by RNA filter hybridization. The region of the gel where tRNAs migrate is shown below the hybridization image to indicate the amount of RNAs loaded.

(b) *SUP* RNA levels in inflorescences of wild-type and *35S::AP2m3* plants as determined by RT-PCR. *UBQ* RNA serves as an internal control. Thirty-five and 25 cycles of PCR are shown for *SUP* and *UBQ*, respectively, although shorter and longer cycles were performed for each gene to ensure that these numbers of cycles did not result in saturation of the reactions. -RT, no reverse transcription.

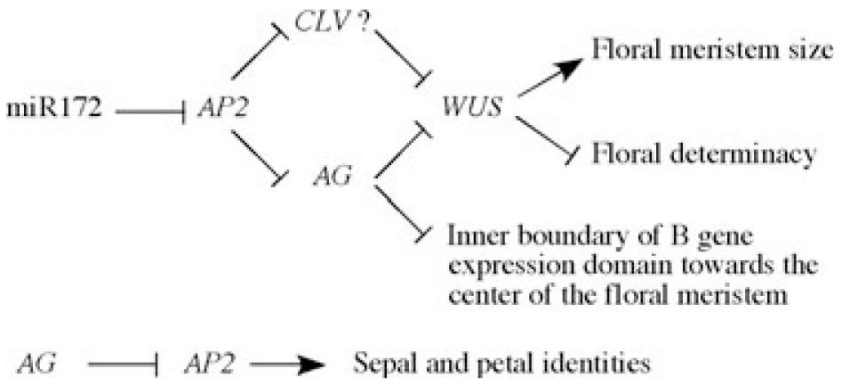

Figure 6.

A diagram summarizing the functions of miR172 and *AG* in flower development as negative regulators of *AP2*.

miR172-mediated repression of *AP2* acts through an *AG*-dependent pathway to promote floral determinacy and to define the inner boundary of the B gene expression domain. miR172-mediated repression of *AP2* also acts in an *AG*-independent, perhaps the *CLV*, pathway to restrict floral meristem size. *AG* antagonizes *AP2* to prevent sepal and petal identities in the inner two whorls. Note that miR172-mediated repression of *AP2* does not play a role in specification of organ identity.