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APETALA2 antagonizes the transcriptional activity of AGAMOUS in regulating floral stem cells in Arabidopsis thaliana

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Summary

- APETALA2 (AP2) is best known for its function in the outer two floral whorls, where it specifies the identities of sepals and petals by restricting the expression of AGAMOUS (AG) to the inner two whors in Arabidopsis thaliana. Here, we describe a role of AP2 in promoting the maintenance of floral stem cell fate, not by repressing AG transcription, but by antagonizing AG activity in the center of the flower.
- We performed a genetic screen with ag-10 plants, which exhibit a weak floral determinacy defect, and isolated a mutant with a strong floral determinacy defect. This mutant was found to harbor another mutation in AG and was named ag-11. We performed a genetic screen in the ag-11 background to isolate mutations that suppress the floral determinacy defect. Two suppressor mutants were found to harbor mutations in AP2.
- While AG is known to shut down the expression of the stem cell maintenance gene WUSCHEL (WUS) to terminate floral stem cell fate, AP2 promotes the expression of WUS.
- AP2 does not repress the transcription of AG in the inner two whors, but instead counteracts AG activity.

Introduction

The angiosperm flower is an innovation that supports sexual reproduction and consists of four types of floral organ: sepal, petal, stamen, and carpel. A flower is formed from a group of undifferentiated cells known as the floral meristem. Like the shoot apical meristem (SAM), floral meristems harbor stem cells in the central zone and descendants of the stem cells that give rise to organ primordia in the peripheral zone. In both the SAM and floral meristems, a small number of cells that express the WUSCHEL (WUS) gene lie underneath the stem cells (Mayer et al., 1998; Schoof et al., 2000). The WUS protein moves out of the cells that express the gene and forms a gradient towards the stem cells, where it activates the expression of the stem cell gene CLAVATA3 to maintain stem cell fate (Yadav et al., 2011; Daum et al., 2014).

Unlike the SAM, which maintains stem cells throughout the life of a plant, floral meristems are determinate insofar as the stem cells are only active for a defined period during which the floral organ primordia are formed. When the final floral organ primordia, carpel primordia, are formed, the floral stem cells cease to be maintained, as evidenced by the absence of subsequent organ primordia and the cessation of WUS expression. The timing of floral stem cell fate termination is tightly coupled with organ formation. While a wild-type Arabidopsis thaliana flower consists of four sepals, four petals, six stamens and two carpels, wus null mutant flowers have four sepals, four petals and a single stamen as a result of the premature termination of floral stem cell fate (Laux et al., 1996). In contrast, failure to terminate the floral stem cells results in additional floral organs or even flowers internal to the fourth whorl. Many genes, such as AGAMOUS (AG), CRABS CLAW (CRC), KNUCKLES (KNU), ULTRAPETALAL1 (ULT1), CURLY LEAF (CLF), AUXIN RESPONSE FACTOR3 (ARF3), POWERDRESS (PWR)

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and MICRONORNA172d (MIR172d), promote the termination of floral stem cell fate (Bowman et al., 1989; Bowman & Smyth, 1999; Carles et al., 2004; Zhao et al., 2007; Prunet et al., 2008; Sun et al., 2009; Liu et al., 2011, 2014a,b; Yumul et al., 2013). The central role of the MADS domain protein AG in conferring floral determinacy is well established, as loss-of-function ag mutants continually produce floral organs, resulting in a flowers-within-flower phenotype (Bowman et al., 1989). Mutations in the other genes result in weaker floral determinacy defects or only result in floral determinacy defects in combination with other mutations. AG promotes floral determinacy by shutting off WUS expression at stage 6 of flower development (Lenhard et al., 2001; Lohmann et al., 2001), both directly at the WUS locus and indirectly through its target gene KNU (Sun et al., 2009; Liu et al., 2011).

In addition to its critical role in floral determinacy, AG is a master regulator in floral organ identity specification. Together with other MADS domain proteins, AG specifies stamen identity in the third whorl and carpel identity in the fourth whorl (Bowman et al., 1991). In ag null mutants, stamens and carpels are replaced by petals and sepals, respectively (Bowman et al., 1989). AG expression is restricted to the inner two floral whorls by APETALA2 (AP2) (Drews et al., 1991), which encodes an AP2 domain transcription factor (Jofuku et al., 1994). In ap2 loss-of-function mutants, the ectopic expression of AG in the outer two whorls results in the transformation of sepals and petals into reproductive organs (Bowman et al., 1991; Drews et al., 1991). AP2 also plays a role in stem cell maintenance in the SAM independent of its function in the repression of AG expression (Wurschum et al., 2006). AP2, a target of miR172 (Aukerman & Sakai, 2003; Chen, 2004), has also been implicated in the control of floral stem cells. The expression of an miR172-resistant version of AP2 driven by its own promoter results in prolonged WUS expression and an indeterminate flower (Zhao et al., 2007). However, a role of AP2 in promoting floral stem cell fate is not obvious in ap2 loss-of-function mutants, which have fewer floral organs in the outer three whorls but the normal number of carpels (Bowman et al., 1989, 1991). The reduced organ number in the outer three whorls is probably linked to AP2’s role in repressing AG expression, as ap2 ag double mutants have normal numbers of organs in the outer three whorls (Bowman et al., 1989, 1991). Thus, there is no clear loss-of-function genetic evidence supporting a role of AP2 in maintaining floral stem cells in the center of the flower.

Through its function as a master regulator of both organ identity and floral determinacy, AG coordinates various cell fate decisions in flower development. However, this raises a logistical problem: while AG is expressed in the inner two whorls from stage 3 and onward to specify the identities of the reproductive organs, its repression of WUS must not commence until stage 6 when the carpel primordia have formed. A timing mechanism involving the delayed activation of KNU by AG at stage 6 has been described (Sun et al., 2009, 2014), but it remains unknown whether other factors contribute to the timing of KNU expression. One possibility is that AG’s activation of KNU and/or repression of WUS is kept in check by a negative regulator. Here we show that AP2 antagonizes AG in the regulation of KNU, WUS and many early floral patterning genes. In this study, we isolated an intermediate-strength ag allele, ag-11, which compromises the floral determinacy but not the organ identity functions of AG. We then performed a genetic screen in the ag-11 background and isolated mutations that suppressed the floral determinacy defect. Two were found to harbor mutations in AP2. We showed that AP2 did not affect the levels of AG transcripts in the inner two floral whorls, but instead antagonized AG in terms of the control of WUS repression. Moreover, AP2 antagonized AG activity in the inner two whorls even when AG was expressed from the cauliflower mosaic virus 35S promoter, which is not controlled by AP2. Therefore, we have uncovered a previously unappreciated function of AP2 as a braking mechanism in the termination of floral stem cell maintenance. Our findings on floral stem cell regulation in the center of the flower also hint at a more complex relationship between AP2 and AG beyond what the canonical model of flower development (i.e. ABC) (Bowman et al., 1991) and updated versions (Wollmann et al., 2010) suggest.

Materials and Methods

Plant material

All mutants and transgenic lines used in this study are in the Arabidopsis thaliana Landsberg erecta (Ler) background. All plants were grown at 23°C under long-day conditions (16 h:8 h, light:dark). ap2-2 (Bowman et al., 1991), ag-10 (Li et al., 2011), ag-10Col (Liu et al., 2011), 35S::AG (Mizukami & Ma, 1992) and 35S::AP2m3 (Chen, 2004) were previously described.

Ethyl methane sulfonate (EMS) mutagenesis and map-based cloning

Approximately 10 000 ag-10 and ag-11 seeds were washed with 0.1% Tween 20 for 15 min, incubated with ethyl methane sulfonate (EMS; 0.2% w/v) for 12 h at room temperature and then washed three times with 10 ml of water (1 h for each wash on a rotator). The treated seeds were grown into M1 plants, and M2 seeds were harvested from the M1 plants for genetic screening. ag-10 enhancers were isolated based on the presence of bulged siliques throughout the plant, and ag-11 suppressors were isolated based on the suppression of the bulged-silique phenotype. The mutants were backcrossed at least two times before further study. For map-based cloning, ag-11 was crossed with ag-10Col to create the mapping population. Simple sequence length polymorphism (SSLP) and cleaved-amplicon polymorphic sequence (CAPS) markers were used to map the mutations. Once ag-11 was mapped to an interval containing the AG locus, AG was selected as a candidate gene for sequencing. For ag-11 suppressors B35 (ag-11 ap2-35) and B43 (ag-11 ap2-43), AP2 was selected as a candidate gene for sequencing based on the similarity of their phenotypes to ap2 loss-of-function mutants.
DNA isolation and genotyping

Two methods, cetyl trimethyl ammonium bromide DNA extraction and ‘quick & dirty’ extraction, were used for DNA isolation as described previously (Dinh et al., 2014). The following primer pairs and enzymes were used for genotyping: ag-10 (JAGp75 and JAGp76; BiX1), ag-11 (ag-11F and ag-11R; Hinfl) and ag-1 (ag-1F and ag-1R; AflI). In each case, the mutations abolish the targeted restriction site. The sequences of the PCR primers are listed in Supporting Information Table S1.

Scanning electron microscopy

Scanning electron microscopy (SEM) imaging was performed with a Hitachi TM-1000 tabletop scanning electron microscope (Hitachi, Tokyo, Japan) according to the manufacturer’s instructions. Samples imaged by the TM-1000 require no special preparation.

In situ hybridization

In situ hybridization was performed as previously described (Chen et al., 2002). To generate the antisense AG probe, the plasmid pCTT565 (Yanofsky et al., 1990) was linearized with HindIII and used as a template for in vitro transcription with T7 RNA polymerase. To generate the WUS probe, a plasmid containing the WUS cDNA was used as the PCR template to generate products containing either the T7 or SP6 promoter sequence. In vitro transcription was performed with either T7 or SP6 RNA polymerase using the purified PCR product as the template to generate the antisense or sense probe, respectively, as previously described (Liu et al., 2011). The PCR primers are listed in Table S1.

RNA isolation and real-time RT-PCR

Total RNA was extracted from inflorescences containing stage 7 and younger flowers using TRI reagent (MRC, Cincinnati, OH, USA), and DNA was removed using DNase I (Roche). RevertAid Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) was used to synthesize cDNA. Quantitative PCR was performed in triplicate using the Bio-Rad CFX-96 Real-time PCR system and iQ SYBR Green Supermix (Bio-Rad). All procedures were performed according to the manufacturers’ instructions. The primers used for real-time reverse transcription–polymerase chain reaction (RT-PCR) are listed in Table S1.

RNA-seq analysis

Total RNA was extracted from inflorescence tissue containing stage 7 and younger flowers using TRI reagent (MRC). Five micrograms of total RNA was used to isolate mRNA using the NEBNext® Poly(A) mRNA Magnetic Isolation Module (NEB, Ipswich, MA, USA). RNA-seq libraries were constructed using the NEBNext® mRNA Library Prep Reagent Set for Illumina (NEB) following the manufacturer’s protocols. Twelve libraries (three replicates for each sample) were pooled and sequenced on an Illumina HiSeq 2500 (Illumina, San Diego, CA, USA) platform at the UCR Genomics Core Facility. Reads that passed Illumina’s quality control filters were further processed. Unique reads were mapped to the Ler genome (Gan et al., 2011) using TopHat v.2.0.13 (Kim et al., 2013), with no mismatches permitted. Reads in gene regions were counted using an in-house Perl script. The expression fold-change of each gene was calculated using the R package DESeq2 (Love et al., 2014) between Ler and ag-11, ag-11 and ag-11 ap2-35, and ag-11 and ag-11 ap2-43, with the threshold for differentially expressed (DE) genes set to a fold-change of 1.5 and a P-value < 0.01. Venn diagrams were generated using VENNY v.2.1 (http://bioinfogp.cnb.csic.es/tools/venny/index.html), and the gene ontology (GO) enrichment analysis of DE genes was performed on the agriGO website (Du et al., 2010). AG and AP2 binding sites were extracted from published chromatin immunoprecipitation (ChIP)-seq data (Yant et al., 2006; Dinh et al., 2014) and mapped to the arabidopsis information resource v.10 (TAIR10; https://www.arabidopsis.org) genome. Genes with binding sites within the gene body and 1000-bp flanking sequences were designated as genes bound by AG or AP2 in vivo.

To assess the statistical significance of the overlap of DE genes between pairs of samples, a chi-squared test was performed with 10 000 iterations of overlap analysis between randomly generated gene sets containing the same numbers of genes as the DE genes.

The RNA-seq data were deposited in GEO under the accession number GSE81205. The link for reviewers: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=uxyjsiqdxbjkp&acc=GSE81205.

Western blotting

Western blotting was performed as previously described (Liu et al., 2011). One hundred milligrams of inflorescence tissue from each sample was ground in liquid nitrogen and homogenized in 2× sodium dodecyl sulfate (SDS) sample buffer (0.5 M Tris-HCl, pH 6.8, 4.4% (w/v) SDS, 20% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, and bromophenol blue). The samples were boiled for 6 min, cooled on ice for 10 min and centrifuged at 16 000 g for 5 min at 4°C to precipitate insoluble material. Proteins in the supernatant were resolved on a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel, transferred to a nitrocellulose membrane and probed with anti-AG (Liu et al., 2011), anti-AP2 (Mlotshwa et al., 2006) and anti-HSC70 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. Signal development was performed with the ECL+Plus Western Blotting system (GE Healthcare, Pasadena, CA, USA) and by exposure of the membrane to X-ray film (Denville, Holliston, MA, USA).

Results

Isolation of ag-11, an ag allele that uncouples the organ identify and floral determinacy functions of AG

We previously reported the isolation and characterization of the ag-10 mutant, which harbors a point mutation resulting in an
E-to-K amino acid substitution in the K domain of AG (Fig. 1a) (Ji et al., 2011). This mutant is the weakest ag allele reported thus far. As in wild type, ag-10 flowers have a full complement of floral organs (Fig. 1b). Most ag-10 flowers are normal in terms of floral determinacy; < 10% of ag-10 flowers have bulged gynoecia with additional floral organs inside, a manifestation of compromised floral determinacy (Ji et al., 2011). We previously mutagenized ag-10 seeds with EMS and conducted a genetic screen for mutants with enhanced floral determinacy defects. Mutants in which nearly all flowers had bulged gynoecia were isolated, and some have been reported (Liu et al., 2011, 2014a,b; Yumul et al., 2013).

Here, we report another mutant from this genetic screen. This mutant had bulged gynoecia in nearly all of its flowers (Figs 1c, 2a,b) and elongated gynophores (Fig. 2b), another feature found in many mutants compromised in floral determinacy (Ji et al., 2011). We previously mutagenized ag-10 seeds with EMS and conducted a genetic screen for mutants with enhanced floral determinacy defects. Mutants in which nearly all flowers had bulged gynoecia were isolated, and some have been reported (Liu et al., 2011, 2014a,b; Yumul et al., 2013).

To map this mutation, we crossed the mutant to ag-10Col, in which the ag-10 mutation was introgressed into the Columbia (Col) background. Using markers polymorphic between Ler and Col, we mapped the mutation to the short arm of chromosome 4 to an interval containing the AG locus. Sequencing of the AG gene itself uncovered a G-to-A mutation that causes an R-to-Q amino acid substitution in the K domain (Fig. 1a). This ag allele harboring both the original ag-10 mutation and this new mutation was designated ag-11. To determine whether the phenotype is attributable to the mutations in AG, we performed a genetic complementation test. The ag-11 mutant was crossed with an ag-1 heterozygous plant. In a total of nine F1 plants, five exhibited an intermediate phenotype that was stronger than ag-11 and weaker than ag-1 (Fig. 1e), and the other four resembled wild type (Fig. S1). Molecular genotyping showed that the five plants were ag-1/ag-11, and the four plants were ag-11/+.

**Fig. 1** Diagram of the Arabidopsis thaliana AGAMOUS (AG) gene and phenotypes of ag mutants. (a) Gene diagram of AG and the locations of the mutations in ag-10 and ag-11. AG is the start codon. The gray and black rectangles represent the 5’ and 3’ untranslated regions and coding regions, respectively. The black lines represent intron regions. (b) An ag-10 flower with a slightly enlarged gynoecium. (c) An ag-11 flower with a much more enlarged gynoecium compared with ag-10. (d) An ag-1 flower exhibiting the flower-within-flower phenotype. (e) An ag-11/ag-1 flower with additional organs within the primary carpels, which are unfused and sepaloid. (b–e) Bars, 1 mm.
Mutations in AP2 partially suppress the floral determinacy defect of ag-11

The fertility of ag-11 allowed a genetic screen in this background with relative ease. Theoretically, loss-of-function mutations that suppress the floral determinacy defect of ag-11 should be in genes that promote floral stem cell fate. A major consideration for performing the ag-11 genetic screen was the fact that WUS is the only gene presently known to promote floral stem cell fate. Thus, we mutagenized ag-11 with EMS and screened for mutations that suppressed the floral determinacy defect.
Two mutants (B35 and B43) with longer and thinner gynoecia were isolated (Fig. 2a,b). Backcrosses to ag-11 showed that each mutant harbored a recessive mutation (Fig. 2a). F1 plants from crosses of the two mutants to each other had longer and thinner gynoecia as compared with ag-11 (Fig. 2a,b), suggesting that these two mutants harbored mutations in the same gene. These mutants also had other floral defects, including reduced floral organ numbers in whorls 1 and 3, carpelloid sepals and a lack of petals (Fig. 2a; Table S2). These phenotypes are characteristic of ap2 loss-of-function mutants (Fig. 2a) (Bowman et al., 1991). When B35 and B43 were crossed to ap2-2, the F1 plants resembled ap2-2 plants in overall floral morphology (Fig. 2c), indicating that B35 and B43 harbor mutations in AP2. Sequencing of AP2 in the two mutants revealed G-to-A mutations resulting in premature stop codons in exon 1 of AP2 (Fig. 2d). As the two mutations introduce premature stop codons close to the N-terminus of AP2, it is likely that they are loss-of-function mutations. To determine whether loss of function in AP2 was responsible for suppressing the ag-11 floral determinacy defect, we introduced the loss-of-function ap2 allele, ap2-2 (Bowman et al., 1991), into ag-11. As with B35 and B43, the ag-11 ap2-2 double mutant exhibited an overall floral phenotype similar to that of ap2 mutants (Fig. 2e). The siliques of ag-11 ap2-2 were long and thin; thus, ap2-2 was also able to suppress the bulged siliques phenotype of ag-11 (Fig. 2f). In addition, we conducted a genetic complementation test by crossing B35 and B43 to ag-11 ap2-2. The F1 progenies from each cross all resembled ap2 mutants in overall floral morphology (Fig. 2f,g) and had longer and thinner gynoecia compared with ag-11 (Fig. 2h). We therefore concluded that AP2 loss of function was responsible for the suppression of ag-11. We refer to the two new ap2 alleles as ap2-35 and ap2-43, respectively.

While most ag-11 gynoecia were short and bulged, most ag-11 ap2-35 and ag-11 ap2-43 flowers had longer, thin and straight gynoecia (Fig. 3a). Quantification of the siliques length:width ratio showed that the two ap2 alleles partially suppressed the short-and-bulged siliques phenotype of ag-11 (Fig. 3b). To further characterize the floral determinacy phenotype, we performed longitudinal and cross sections of stage 11 and older flowers. In the wild type, stage 11 flowers had gynoecia with ovules, and the floral meristem was not visible inside the gynoecium at this stage (Fig. 3c). In stage 11 ag-11 flowers, a floral meristem was present within the gynoecium near the base (Fig. 3d). In a cross section, it was obvious that additional floral organs were present inside the fourth whorl gynoecium (Fig. 3g); this was never observed in the wild type (Fig. 3f). In ag-11 ap2-35, 24 out of 26 flowers examined resembled ap2-2 flowers, while two out of 26 flowers examined had floral organs inside the gynoecium (Fig. 3h). These observations show that the two ap2 alleles partially suppressed the ag-11 floral determinacy defect.

AP2 promotes WUS expression without affecting AG transcription in the center of floral meristems

We next sought to determine the molecular basis of the suppression of ag-11 phenotypes by ap2 mutations. We first examined WUS expression by in situ hybridization, as AG-mediated cessation of WUS expression by stage 6 of flower development is responsible for floral determinacy. Consistent with previous observations in wild type (Mayer et al., 1998), WUS expression was detected in stage 3 floral meristems in a small number of cells underneath the stem cells, but WUS expression was not detected in stage 6 or older flowers (Fig. 4a). In ag-11 plants, however, all examined stage 6 and older flowers had WUS expression (n = 12) (Fig. 4b). Prolonged WUS expression is consistent with the continued presence of a floral meristem inside the gynoecium in ag-11 (Fig. 3d). In ag-11 ap2-35, 11 out of 15 stage 6 or older flowers examined resembled wild type in having no WUS expression (Fig. 4c). The remaining four resembled ag-11. Loss of function in AP2 therefore partially suppressed ag-11 in terms of the WUS repression defect; in other words, the prolonged WUS expression in ag-11 flowers required AP2 function.

As AG is the key factor that represses WUS expression in stage 6 floral meristems, we next examined whether AP2’s role in promoting WUS expression was attributable to the ability of AP2 to repress AG expression in the center of the flower. AP2 is known to repress AG transcription in the outer two floral whorls (Drews et al., 1991), but it is thought not to repress AG transcription in the center of the floral meristem, as ectopic AP2 activity in ag-1 was not sufficient to prevent the accumulation of ag-1 transcript in the inner whorls of ag-1 mutant flowers (Gustafson-Brown et al., 1994). First, we assessed whether AG transcript levels were affected by the ag-11 mutation or the ap2 mutations. RNA-seq using inflorescences containing stage 7 and younger flowers from ag-11, ag-11 ap2-35 and ag-11 ap2-43 showed increased AG transcripts in ag-11 ap2-35 and ag-11 ap2-43 compared with ag-11 (Fig. 5c). The increase in AG expression was probably attributable to the known repression of AG transcription in the outer two whorls by AP2. To determine whether AP2 repressed AG transcription in the center of the floral meristem, where floral determinacy takes place, we examined AG expression by in situ hybridization in developing flowers. AG transcripts were present in the center of floral meristems but excluded from sepal primordia in wild type and ag-11 (Fig. 4d,e). In ag-11 ap2-35, AG RNA was detected not only in the center of the floral meristem but also in sepals (Fig. 4f). Although in situ hybridization is not quantitative, AG RNA signals were similar in ag-11 and ag-11 ap2-35 in the center of the floral meristem (Fig. 4f). Therefore, the effect of the ap2 mutations on WUS expression could not be attributed to obvious changes in AG transcription in the center of the floral meristem.

AP2 and AG have opposite transcriptional outputs at the genomic scale

AP2 clearly exerts the opposite effect on WUS expression and floral determinacy compared with AG, and yet it does not appear to affect AG transcription in the center of the floral meristem. This suggests that AP2 antagonizes AG activity, perhaps by preventing AG from acting on its target genes or acting independently on the same target genes to exert the opposite effect. To determine
whether \( AP2 \) and \( AG \) have antagonistic effects on transcription, we examined the gene expression profiles of \( Ler, ag-11, ag-11 \) \( ap2-35 \) and \( ag-11 \) \( ap2-43 \) using RNA-seq. Inflorescences containing stage 7 and younger flowers were used, and three biological replicates were performed. For each genotype, the three biological replicates were highly correlated (Fig. S3a), indicating that the RNA-seq data were reproducible. Using DESeq2, we identified DE genes for each pairwise comparison using fold-change > 1.5 and \( P \)-value < 0.01 as the threshold (Tables S3, S4). Compared with \( Ler \), 248 up-regulated and 109 down-regulated genes were found in \( ag-11 \) (Table S3). As expected, genes involved in plant development, especially flower development, were among the most significantly enriched in the down-regulated genes (Fig. S3c). Intriguingly, genes involved in immune responses and cell death were significantly enriched in the up-regulated genes (Fig. S3b). Eleven of the 248 up-regulated genes and 11 of the 109 down-regulated genes had AG-binding sites, as determined by AG ChiP-seq (O’Maónlaidh et al., 2013), and may thus be direct targets of \( AG \) (Fig. 5a,b; Table S3). The other DE genes may be indirectly regulated by \( AG \).

Remarkably and rather unexpectedly, the differential expression of a large portion of the 248 and 109 genes in \( ag-11 \) was rescued by the two \( ap2 \) mutations. Among the 248 genes showing increased expression in \( ag-11 \) vs \( Ler \), 86 and 69 overlapped with genes with reduced expression in \( ag-11 \) \( ap2-35 \) vs \( ag-11 \) and \( ag-11 \) \( ap2-43 \) vs \( ag-11 \), respectively (Fig. 5a). The overlap was statistically significant (see Table S5 for \( P \)-values). Thus, \( AP2 \) is required for the increased expression of these genes in the \( ag-11 \) mutant. Among the 109 genes showing reduced expression in \( ag-11 \), 35 and 32 overlapped with the genes up-regulated in \( ag-11 \) \( ap2-35 \) vs \( ag-11 \) and \( ag-11 \) \( ap2-43 \) vs \( ag-11 \), respectively (Fig. 5b; see Table S5 for \( P \)-values), indicating that \( AP2 \) is required for the reduced expression of these genes in \( ag-11 \). Among genes co-regulated by \( AG \) and \( AP2 \) were genes with roles in early flower development described in a previous review article (Vaddepalli et al., 2015), and the altered expression of these genes in \( ag-11 \) was restored by the mutations in \( AP2 \) (Fig. 5c). Of particular note was that genes with previously established roles in floral stem cell regulation, such as \( WUS \), \( KNU \), \( PWR \), and
ARF3, were among the genes co-regulated by AG and AP2 in opposite directions (Fig. 5c). These findings show that AP2 antagonizes the transcriptional activity of AG in the control of floral determinacy and perhaps in other aspects of flower development. It is worth noting that a much larger group of genes was affected by the ap2 mutations (ag-11 ap2 vs ag-11) than by ag-11 (ag-11 vs Ler). Ectopic AG expression in the outer two whorls in ap2 mutants could have contributed to a portion of the differentially expressed genes in ag-11 ap2 vs ag-11.

Fig. 5 Antagonistic effects of Arabidopsis thaliana AGAMOUS (AG) and APETALA2 (AP2) on target gene expression as determined by RNA-seq. (a) Venn diagram showing the overlap between genes with increased expression in ag-11 vs Ler and genes with reduced expression in ag-11 ap2-35 vs ag-11 or ag-11 ap2-43 vs ag-11. (b) Venn diagram showing the overlap between genes with reduced expression in ag-11 vs Ler and genes with increased expression in ag-11 ap2-35 vs ag-11 or ag-11 ap2-43. In (a) and (b), genes with AG binding sites as determined by AG chromatin immunoprecipitation (ChIP)-seq (O’Maoridigh et al., 2013) are also shown. The numbers in parentheses represent the numbers of genes with altered expression between the two indicated genotypes. (c) Expression levels of selected differentially expressed (DE) genes in ag-11 vs Ler with known roles in early flower development. The differential expression of these genes in ag-11 was restored by the mutations in AP2. The heatmap was generated with Z-score values derived from Z score = (x – mean)/SD (x being one of the four genotypes; mean and SD being calculated from the four genotypes). (d) Reads per kilobase per million mapped reads (RPKM) values for select AG target genes (genes bound by AG in vivo) in the indicated genotypes. The mean and SD from three biological replicates of RNA-seq are shown. The asterisks indicate statistically significant changes relative to ag-11. Fold change ≥ 1.5 and P-value < 0.01. CLV1, CLAVATA1, DAD1, DEFECTIVE ANther DEHISCENCE 1; TMK1, TRANSMEMBRANE KINASE 1; PWR, POWERDRESS; AP3, APETALA3; TOP12, DNA TOPOISOMERASE I ALPHA; ROP6, Rho-like GTPase 6; AP1, APETALA1; ELA1, EU- LIKE P450 A1; TIR1, TRANSPORT INHIBITOR RESPONSE 1; RIC1, ROP-INTERACTIVE CRIB MOTIF-CONTAINING PROTEIN 1; WUS, WUSCHEL; CLV3, CLAVATA3; D6PK, D6 PROTEIN KINASE; SEP3, SEPALATA3; LFY3, LEAFY 3; AIL6, ANTIGUMENTA-LIKE 6; LCR, LEAF CURLING RESPONSIVENESS; SHP2, SHATTERPROOF 2; ANT, ANTIGUMENTA; QKY, QUIRKY; SCM, SCRAMBLED; ABP1, AUXIN BINDING PROTEIN 1; ARF5, AUXIN RESPONSE FACTOR 5; ARF3, AUXIN RESPONSE FACTOR 3; PID, PINOID; PIN1, PIN-FORMED 1; KNU, KNUCKLES; AG, AGAMOUS; MAB4, MACCHI-BOU 4; CRC, CRABS CLAW; SHP1, SHATTERPROOF 1; SVB, SMALLER WITH VARIABLE BRANCHES; SARD1, SAR DEFICIENT 1; LSH3, LIGHT SENSITIVE HYPOCOTYLS 3; CIPK12, CBL-INTERACTING PROTEIN KINASE 12; BCAT2, BRANCHED-CHAIN AMINO ACID TRANSAMINASE 2; DMR6, DOWNY MILDEW RESISTANT 6; RPT2, ROOT PHOTOTROPISM 2; MYC3, MYC TRANSCRIPTION FACTOR 3.
We next examined whether AP2 has an effect on direct targets of AG. Among the 11 up-regulated and 11 down-regulated direct AG targets showing differential expression in ag-11 vs Ler, five of each category showed differential expression in the two ap2 mutants in the opposite direction (Fig. 5a,b). That is, the altered expression of these AG target genes in ag-11 was restored by the mutations in AP2 (some of the genes are shown in Fig. 5d). These findings indicate that AP2 exerts the opposite effect on AG direct target genes. We asked whether these AG target genes are also potential AP2 direct targets. Using published AP2 ChIP-seq data (Yant et al., 2010), we found that two (SHATTERPROOF 1 (SHP1) and SHP2) of the five down-regulated AG targets in ag-11 were bound by AP2 in vivo and therefore potentially direct targets of AP2 (Table S3). Thus, AG and AP2 probably have direct and antagonistic effects on the transcription of these genes.
**AP2** antagonizes AG activity in controlling floral determinacy

To further establish that AP2 antagonizes AG activity rather than inhibiting AG expression, we examined whether AP2 affects floral determinacy when AG transcription is rendered independent of AP2. AP2 is known to repress AG transcription in the outer two whorls through the large second intron of AG (Bomblies et al., 1999; Deyholos & Sieburth, 2000; Dinh et al., 2012). We expressed AG cDNA (thus devoid of the second intron) from the cauliflower mosaic virus 35S promoter. A transgenic line showing higher levels of AG RNA (Fig. 6a) and protein (Fig. 6b) was chosen for further analysis. This line exhibited loss of floral determinacy (Fig. 6c), as previously reported for the overexpression of miR172-resistant AP2 (Chen, 2004; Zhao et al., 2007). To test whether AP2 is functional in the presence of 35S::AG, we crossed 35S::AP2m3 to 35S::AG. In 35S::AG 35S::AP2m3 flowers, there were higher levels of both AP2 and AG proteins than in wild type. The gynoecia of 35S::AG 35S::AP2m3 flowers were bulged and present on top of elongated gynophores; the phenotype resembled that of the 35S::AP2m3 line but differed from that of the 35S::AG line used in the cross (Fig. 6c). Therefore, AP2 was able to antagonize the activity of AG even when AG was not under transcriptional repression by AP2.

**KNU** is the only gene known to act downstream of AG to promote floral determinacy (Sun et al., 2009). AG activates KNU expression at stage 6, and KNU in turn represses WUS expression (Sun et al., 2009). We investigated whether AP2 also regulates KNU expression and, if so, whether it exerts the opposite effect to that of AG. We examined KNU RNA levels in the ag-11, ag-11 ap2-35 and ag-11 ap2-43 RNA-seq data. KNU transcript levels were increased in ag-11 ap2-35 and ag-11 ap2-43 relative to ag-11 (Fig. 5c), indicating that AP2 represses KNU expression. We chose for further analysis. This line exhibited loss of floral determinacy (Fig. 6c), as previously reported for the overexpression of miR172-resistant AP2 (Chen, 2004; Zhao et al., 2007). To test whether AP2 is functional in the presence of 35S::AG, we crossed 35S::AP2m3 to 35S::AG. In 35S::AG 35S::AP2m3 flowers, there were higher levels of both AP2 and AG proteins than in wild type. The gynoecia of 35S::AG 35S::AP2m3 flowers were bulged and present on top of elongated gynophores; the phenotype resembled that of the 35S::AP2m3 line but differed from that of the 35S::AG line used in the cross (Fig. 6c). Therefore, AP2 was able to antagonize the activity of AG even when AG was not under transcriptional repression by AP2.

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also compared KNU expression in wild type, ap2-2 and 35S::AP2m3. Consistent with a repressive role of AP2 on KNU expression, KNU expression was higher in ap2-2 and dramatically reduced in 35S::AP2m3 relative to wild type (Fig. 6d). To address whether AP2 antagonizes AG in terms of KNU expression regulation, we compared KNU expression in 35S::AG and 35S::AG 35S::AP2m3. KNU expression was similarly low in 35S::AP2m3 and 35S::AG 35S::AP2m3, suggesting that AP2 overexpression overcame the presence of high levels of AG protein (Fig. 6d). These findings show that KNU, a key target of AG in floral determinacy, is also regulated by AP2 but in the opposite way.

Discussion
Efforts to understand the mechanisms of stem cell regulation in plants have largely focused on the SAM, which maintains its stem cell population throughout the life of a plant. Floral meristems undergo genetically programmed termination of stem cell fate and this process is coordinated with other programs of flower development, such as floral organ formation and fruit development. Thus, floral meristems offer a great model with which to understand stem cell maintenance and termination as well as the interplay between stem cell activity and other developmental processes. From a practical point of view, adjusting the timing of stem cell termination is coupled to carpel primordia formation.

In A. thaliana, the termination of floral stem cell fate is accomplished by the repression of the stem cell maintenance gene WUS by AG at stage 6 of flower development when the carpel primordia have been formed. A feed-forward loop consisting of AG, its target KNU, and WUS, a target of both AG and KNU, is probably at work to terminate floral stem cell fate (Fig. 7). In addition to AG and KNU, many other genes have been shown to participate in the repression of WUS (Bowman & Smyth, 1999; Carles et al., 2004; Zhao et al., 2007; Prunet et al., 2008; Ji et al., 2011; Liu et al., 2011; 2014a,b; Yumul et al., 2013). By contrast, how WUS expression is maintained in flower development is largely unknown. We showed that overexpression of AP2 in flowers, achieved through the expression of miR172-resistant AP2, leads to prolonged WUS expression and indeterminate floral meristems (Zhao et al., 2007). However, this evidence based on AP2 overexpression was insufficient to establish a normal function of AP2 in floral determinacy and maintenance of WUS expression.

In this study, we provide loss-of-function evidence that AP2 maintains WUS expression in the floral meristem and promotes floral stem cell fate. Two ap2 alleles with early stop codons were isolated as suppressors of the floral determinacy defects of ag-11. The prolonged WUS expression in ag-11 was suppressed by the two ap2 mutations, suggesting that AP2 promotes WUS expression. AP2 is well known for its role in floral organ identity specification in the outer two floral whorls, where it represses AG transcription and promotes the formation of sepals and petals. Here, we show that AP2 also has a role in the center of the flower, where it promotes stem cell maintenance. Another study also revealed a role of AP2 in fruit development (Ripoll et al., 2011). Therefore, AP2’s function in flower development is not restricted to the outer two whors.

Another important conclusion from this work is that AP2 antagonizes AG in floral determinacy not through the repression of AG transcription, as it does in the outer two whors. We show that the two ap2 loss-of-function alleles suppress ag-11 without affecting AG RNA levels in the center of the flower where floral stem cells reside. We also show that AP2 overexpression can compromise floral determinacy even when AG is overexpressed from the 35S promoter. Therefore, AP2 must be able to antagonize the function of AG either directly or indirectly. We show that AP2 reduces the expression of KNU even in the 35S::AG background. Therefore, AP2 could exert its antagonistic effects on AG by repressing the AG target gene KNU. AP2 could serve as a brake in the feed-forward loop consisting of AG, KNU and WUS (Fig. 7). Note that KNU is a direct target of AG (Sun et al., 2009), but it is not known whether AP2 acts directly on the KNU locus.

Finally, this study revealed a remarkable and rather unexpected antagonistic relationship between AG and AP2 at the genomic scale. The altered expression of 25–35% of the 357 DE genes in ag-11 was affected in the opposite direction by the two ap2 loss-of-function mutations. Among the 357 genes, 22 are bound by AG in vivo and are probably direct AG targets. Ten of the 22 AG direct target genes were found to be co-regulated by AG and AP2 in opposite directions. Therefore, AP2 has a profound and previously unappreciated effect on AG’s transcriptional output.

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Author contributions
X.C., L.X. and Z.G. planned and designed the research. Z.H., T.S., B. Z., R.E.Y. and X.L. performed experiments. C.Y. analyzed genomics data. X.C. and Z.H. wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Genotyping of the ag-1 and ag-11 mutations in the F1 progeny of the cross ag-11 × ag-11/+

Fig. S2 Phenotypes of the indicated genotypes.

Fig. S3 RNA-seq analysis of wild type (Ler), ag-11, ag-11 ap2-35, and ag-11 ap2-43.

Table S1 Sequences of oligonucleotides used in this study

Table S2 Floral organ counts and quantification of floral determinacy defects

Table S3 Genes with differential expression between ag-11 and Ler

Table S4 Genes with differential expression between ag-11 ap2-35 and ag-11 and between ag-11 ap2-43 and ag-11

Table S5 The P-value of overlaps between DE genes

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