APETALA2 negatively regulates multiple floral organ identity genes in Arabidopsis by recruiting the co-repressor TOPLESS and the histone deacetylase HDA19

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SUMMARY
The development and coordination of complex tissues in eukaryotes requires precise spatial control of fate-specifying genes. Although investigations of such control have traditionally focused on mechanisms of transcriptional activation, transcriptional repression has emerged as being equally important in the establishment of gene expression territories. In the angiosperm flower, specification of lateral organ fate relies on the spatial regulation of the ABC floral organ identity genes. Our understanding of how the boundaries of these expression domains are controlled is not complete. Here, we report that the A-class organ identity gene APETALA2 (AP2), which is known to repress the C-class gene AGAMOUS, also regulates the expression borders of the B-class genes APETALA3 and PISTILLATA, and the E-class gene SEPALLATA3. We show that AP2 represses its target genes by physically recruiting the co-repressor TOPLESS and the histone deacetylase HDA19. These results demonstrate that AP2 plays a broad role in flower development by controlling the expression domains of numerous floral organ identity genes.

KEY WORDS: APETALA2, Floral development, Transcriptional repression, Arabidopsis

INTRODUCTION
In multicellular organisms, complex functional structures often require precise spatial arrangements of individual tissues. How the identities of these different tissues are established is a central question in developmental biology, and usually involves domain-specific expression patterns of fate-specifying genes. Although early studies on eukaryotic gene expression focused on mechanisms of transcriptional activation, it has become clear that transcriptional repression is equally important in the regulation of cell identity (Gray and Levine, 1996). Gene expression boundaries underlying many patterning processes rely on DNA-binding transcription factors that negatively regulate target genes by interacting with co-repressors. These co-repressors recruit proteins that confer active transcriptional repression, including chromatin-modifying factors (Courey and Jia, 2001). Co-repressors of the Groucho (Gro)/Transducin-like enhancer of split (TLE) family are well characterized in animal systems and have been implicated in a myriad of developmental events, including Drosophila dorsoventral patterning and vertebrate anteroposterior patterning (Buscariel and Stifani, 2007).

In plants, the flower is a useful model for investigating transcriptional mechanisms of fate specification. It comprises four types of lateral organs (sepals, petals, stamens and carpels) arranged in concentric whorls in eudicots (numbered 1 to 4 from the peripheral sepals to the central carpels). Floral organ identity genes specify the fate of each whorl, and their misregulation can lead to hemeotic conversions of one organ type to another. Such conversions are produced by a number of classic mutants in model angiosperm species such as Arabidopsis and Antirrhinum, the study of which led to the establishment of the ABC model of flower development over two decades ago (Bowman et al., 1991; Coen and Meyerowitz, 1991). This model posits that three functions, termed A, B and C, act alone or in combination to specify the identities of the floral lateral organs. A-function specifies sepal fate, A and B together confer petal identity, B and C jointly specify stamen fate, and C confers carpel identity in the central whorl. Additionally, A and C were hypothesized to operate antagonistically by restricting each other’s functional domains. In Arabidopsis, these activities were originally attributed to four genes based on genetic analyses: APETALA2 (AP2) provided A-function; APETALA3 (AP3) and PISTILLATA (PI) conferred B-function; and AGAMOUS (AG) provided C-function. APETALA1 (AP1) was later identified as another A-function gene (Bowman et al., 1993), and more recently, an E-function has been defined by the SEPALLATA (SEP) E-family (Pelaz et al., 2000; Ditta et al., 2004). Like the ABC factors (with the exception of AP2), SEPs are MADS-domain proteins, and they facilitate the formation of ABCE transcriptional complexes that specify floral organ fate (Honma and Goto, 2001). Quadruple loss-of-function sep mutants have flowers consisting solely of leaf-like organs (Ditta et al., 2004), and ectopic expression of SEP and ABC genes is sufficient to convert leaves into floral organs (Honma and Goto, 2001; Pelaz et al., 2001). Unlike the ABC genes, expression of which is generally confined to two neighboring whorls, most SEP genes are expressed in all floral organ primordia. SEP3 is an exception, as its expression is mainly restricted to the inner three whorls (Mandel and Yanofsky, 1998).

Although ABCE genes are initially activated by floral meristem identity genes (Weigel et al., 1992; Bowman et al., 1993; Weigel and Meyerowitz, 1993; Lee et al., 1997), their functional domains are refined by cadastral regulators that establish the boundaries of their expression. AP2 restricts AG expression to whorls 3 and 4, and in strong ap2 mutants, AG spreads throughout the flower resulting in carpel-like whorl 1 organs and loss of whorl 2 organs (Bowman et al., 1991; Drews et al., 1991). Other factors have been...
shown to contribute to the spatial restriction of AG (Byzova et al., 1999; Bao et al., 2004; Takeda et al., 2004; Kriek et al., 2006), including the transcriptional co-repressor LEUNIG (LUG) and its interacting partner SEUSS (SEU) (Liu and Meyerowitz, 1995; Conner and Liu, 2000; Franks et al., 2002). SUPERMAN (SUP), a C2H2-type zinc finger protein, is necessary for maintaining the inner boundary of B-class gene expression between whorls 3 and 4, as AP3 expression expands into the central region of sup flowers, producing ectopic stamens at the expense of carpels (Schultz et al., 1991; Bowman et al., 1992; Sakai et al., 1995). A small RNA (miR172) that regulates AP2 also aids in the establishment of this border, as overexpression of an miRNA-resistant AP2 transgene results in expansion of AP3 and PI towards the center of the floral meristem (Park et al., 2002; Aukerman and Sakai, 2003; Chen, 2004; Zhao et al., 2007). Despite these advances, many questions regarding the spatial control of the ABC gene remain. For instance, the mechanisms underlying many of these regulatory relationships, including the inhibition of AG by AP2, have yet to be fully characterized. Additionally, it is unknown whether cadastral regulators directly restrict the peripheral expansion of B- and E-class (SEP3) genes in the outermost whorl.

Here, we report one molecular mechanism underlying the repression of AG by AP2, a genetic relationship that contributed to one of the original principles of the ABC model (antagonism of C-by A-function). Specifically, we show that AP2 directly represses AG by recruiting a transcriptional co-repressor and a histone deacetylase. This AP2 repressor complex also targets B- and E-class genes to restrict their expression in whorl 1, elucidating new regulatory functions for AP2 not previously defined in the ABC model. Collectively, these results show that AP2 plays a broad role in flower development, spatially controlling the expression domains of multiple floral organ identity genes.

MATERIALS AND METHODS

Plant material

Plants were grown on soil in greenhouse conditions under a 16-hour light/8-hour dark cycle. The Landsberg erecta (Ler) ecotype of Arabidopsis was used as wild type. Genetic analyses utilized the previously described mutants tpl-1 (Long et al., 2002), tpl-2, tpl1, tpl3, tpl4, hda19-1 (Long et al., 2006), ap2-2, pi-1, ag-1 (Bowman et al., 1989), ap3-3 (Jack et al., 1992) and sep3-2 (Pelaz et al., 2000). tpl2 is a T-DNA insertion allele (SALK_112730) (Alonso et al., 2003). Owing to the unavailability of Ler sep3 alleles, tpl-1 was introgressed into the Col-0 ecotype and then crossed to the sep3-2 mutant for genetic analyses. To genotype plants for tpl-1, hda19-1, ap2-2, ap3-3, pi-1, ag-1 and sep3-2 mutant alleles, DNA extractions were used as template in PCR with primers given in supplementary material Table S1.

RNA in situ hybridization

RNA in situ hybridizations with digoxigenin-labeled riboprobes were performed as described at http://phio.salk.edu/phbcln/situ_protocol.html. Probes used for expression of AP3 (Jack et al., 1992), PI (Goto and Meyerowitz, 1994), AG (Yanofsky et al., 1990) and HDA19 (Long et al., 2006) have been detailed previously. The entire coding region of TPL was used as probe. Three different SEP3 probes were used and all produced similar expression patterns. These included the full-length coding sequence and two probes previously described (Mandel and Yanofsky, 1998; Liu et al., 2009). Stages attributed to flowers, as described in figure legends, are based on standard convention (Smyth et al., 1990).

Protein interaction analyses

Yeast two-hybrid (Y2H) assays were performed with yeast strain MaV203 using Invitrogen ProQuest expression vectors and product protocols. Semi-in vivo pull-down experiments with TPL and TPL-N176H GST fusions, including expression and purification of reconstituent proteins, were carried out as previously reported (Szemenyi et al., 2008) with minor modifications. Specifically, unopened floral buds from stages 1 to 12 (Smyth et al., 1990), along with the inflorescence meristem, were harvested from stably transformed Arabidopsis lines and processed to produce plant lysates. TPL Y2H and GST fusion constructs have been described (Szemenyi et al., 2008). PCR-generated AP2 CDNAs were cloned into SalI-NotI sites of pDBLeu and pEXP-ADS02 Y2H vectors (Invitrogen) or into XbaI-SalI sites of GST expression vector pGEX-KG. Conserved leucines within the EAR motif of AP2 were converted to alanines (CTCGATTTCGGTTG to gcCGATgcGAGCcG) by site-directed mutagenesis. Bimolecular fluorescence complementation (BiFC) experiments were performed in a tobacco transient expression system as previously detailed (Szemenyi et al., 2008). A full-length HDA19 genomic clone (Xcol-KpnI) was used to generate BiFC expression constructs.

Chromatin immunoprecipitation (ChIP) and western blots

ChIP experiments were performed as previously described (Bowler et al., 2004; Smith and Long, 2010) with the following modifications. Starting material consisted of unopened floral buds from stages 1 to 12 (Smyth et al., 1990), along with the inflorescence meristem. Protein G Dynabeads (Invitrogen) were used with anti-HA (Covance HA.11), anti-GFP (Abcam ab290), or anti-H4K16Ac (Active Motif 39167) antibodies in immunoprecipitations. Tissues expressing epitope-tagged HDA19 were pre-treated with 1.5 mM ethylene glycol bis(succinimidylsuccinate) (EGS) (Thermo Scientific) for 1 hour under vacuum. Pre-treatment of non-transgenic wild-type (Ler) tissue with EGS did not significantly affect ChIP enrichment values relative to non-EGS-treated control tissue. Therefore, EGS-treated Ler control values are not presented in Fig. 2L, Fig. 3O, Fig. 5L and supplementary material Fig. S4. Anti-H3K9Ac (Abcam ab4441), anti-H4K5Ac (AbD Serotec AP962) and anti-H3 (Abcam ab1791) antibodies were used for western blotting. Western signal intensities were quantified using ImageJ software (National Institutes of Health, http://imagej.nih.gov/ij/).

Real-time PCR

Real-time PCR on ChIP samples was performed with a Bio-Rad CFX96 Real-time PCR system using Bio-Rad SsoFast EvaGreen Supermix reagents according to manufacturer protocols. Data analysis, including calculation of primer pair reaction efficiencies and Ct values, was carried out by Bio-Rad CFX Manager software. Enrichment was calculated as a ratio of the signal from ChIP samples to that from input samples. Fold enrichment was presented based on normalization with ACT2 data.

Transgenic plant lines

Arabidopsis transgenic lines were generated by the Agrobacterium-mediated floral dip method into the Ler ecotype (Clough and Bent, 1998). HDA19p::HDA19-HA was made using a PCR-generated HDA19 genomic clone (including 716 bp of promoter sequence) that was introduced into the Nole site of plasmid pBJ36 (Eshed et al., 2001) upstream of a 6xHA epitope tag. This fusion gene was subcloned into the Nol site of binary vector pART27 (Gleave, 1992), transformed into hda19-1, and fully complemented the mutant. AP2 and AP2mEAR CDNAs were cloned as PCR-generated SalI-KpnI fragments downstream of the 2x35S promoter and upstream of GFP in vector PBH36. Each AP2 fusion was subcloned into a sulfadiazine resistance binary vector (Heisler et al., 2010) and transformed into hda19-1 HDA19p::HDA19-HA for use in pull-down assays. The TPL::TPL-HA transgenic line employed in pull-down and ChIP experiments has been described (Szemenyi et al., 2008). TPL::TPL-GFP, HDA19p::HDA19-GFP and AP2::AP2xYFP-3xHA transgenes used in ChIP assays have been published previously (Long et al., 2006; Wollmann et al., 2010).

TPL::TPL-AP2DBs is a translational fusion between a full-length TPL cDNA and the coding sequence for the DNA-binding domains of AP2, followed by the recognition sequence for the small RNA mir172. This transgene was cloned into vector pB336 behind 4.13 kb of TPL promoter sequence and upstream of GFP. The TPL coding sequence was removed from this construct and a start codon was introduced into AP2DBs to produce the transgene TPL::TPL-AP2DBs. PCR was used to generate AP2DBs fragments with Smal restriction sites at their termini to facilitate

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cloning (primer sequences in supplementary material Table S1). Both transgenes were introduced into the NotI site of the binary vector pMLBART (Eshed et al., 2001) for plant transformation.

To generate 2x35Sp::SEP3, a full-length SEP3 cDNA was amplified by PCR (as a ClaI-BamHI fragment) and cloned downstream of a 2x35S promoter and upstream of YFP-3xHA in plasmid pBJ36. This transgene was transferred into the NotI site of binary vector pMLBART and transformed into a Ler wild-type background.

Transcriptional repression assay

The transcriptional repression assay employing the β-glucuronidase (GUS) reporter was performed as previously described (Szemenyei et al., 2008). 2x35Sp::AP2-GAL4DB-3xHA was generated by fusing a full-length AP2 cDNA clone (SalI-KpnI) to GAL4DB-3xHA in pBJ36. This transgene was then transferred into the NotI site of binary vector pMLBART and transformed into a Ler wild-type background.

Microscopy

RNA in situ hybridizations were imaged using a Leica DM5000B compound microscope. Whole flowers and floral organs were imaged with a Leica MZ FLIII stereomicroscope. Scanning electron micrographs were acquired using a Zeiss EVO LS 15 analytical environmental scanning electron microscope.

RESULTS

APETALA2 physically interacts with a co-repressor and histone deacetylase

Although it is well established that AP2 represses AG, the molecular mechanism behind this control is not known (Drews et al., 1991; Yant et al., 2010). Using a yeast two-hybrid (Y2H) screen, we determined that AP2 physically interacts with the Gro/Tup1-type co-repressor TOPLESS (TPL), a result confirmed in a recent report (Causier et al., 2012). This interaction depended on the C-Terminal to Lissencephaly Homology (CTLH) domain of TPL (Fig. 1A), which is required for other reported TPL protein interactions (Szemenyei et al., 2008; Gallavotti et al., 2010). AP2 contains a consensus ERF-associated amphiphilic repression (EAR) motif (LxLxL) (Fig. 1A) (Ohta et al., 2001; Kagale et al., 2010), which has been implicated in the physical recruitment of TPL by various proteins (Szemenyei et al., 2008; Gallavotti et al., 2010; Pauwels et al., 2010). Mutation of the AP2 EAR motif (mEAR) abolished interaction with TPL in yeast, showing that this motif is necessary for AP2-TPL association (Fig. 1A). The tpl-1 missense mutation (N176H), which acts as a dominant negative for the TOPLESS RELATED (TPR) family (Long et al., 2006), also disrupted binding with AP2 in yeast (Fig. 1A). We verified these results using semi-in vivo pull-down assays (Szemenyei et al., 2008), testing the ability of recombinant GST-tagged TPL or TPL N176H amino-terminal fragments to bind GFP fusions of wild-type AP2 or AP2mEAR expressed in stable transgenic Arabidopsis lines. GST-N-TPL, but not GST-N-TPL N176H, interacted with wild-type AP2-GFP (Fig. 1B), whereas both GST-TPL variants were able to bind to full-length TPL (supplementary material Fig. S1A) (Szemenyei et al., 2008). Consistent with our Y2H data, GST-N-TPL was unable to pull-down AP2mEAR (Fig. 1B).
Co-repressors commonly recruit chromatin-remodeling factors to actively repress gene expression, and prior genetic analyses implicated the RPD3-like HISTONE DEACETYLASE 19 (HDA19) in TPL-dependent transcriptional regulation (Long et al., 2006). Therefore, we assessed the ability of TPL to pull-down HDA19-HA, implicated the RPD3-like HISTONE DEACETYLASE 19 (HDA19) to regulate the C-class gene AG, which was expressed in the same transgenic Arabidopsis lines. We next sought to determine whether AP2 recruits TPL and HDA19 to regulate the C-class gene AG in outer whorl floral organs. Therefore, we assessed the ability of TPL to pull-down HDA19-HA, implicated the RPD3-like HISTONE DEACETYLASE 19 (HDA19) to regulate the C-class gene AG, which was expressed in the same transgenic Arabidopsis lines. We next sought to determine whether AP2 recruits TPL and HDA19 to regulate the C-class gene AG in outer whorl floral organs. (A) Wild-type Arabidopsis flower composed of four sepals (se), four petals (p), six stamens (st) and two fused carpels (c). (B-D) Mutant flowers exhibiting carpel-like whorl 1 organs (arrowheads) with stigmatic tissue at their margins. Arrow in B designates stamen-like identity. (E-H) AG in situ hybridizations on stage 7-8 flowers. (E) Wild-type flower with expression restricted to stamen and carpel primordia. (F-H) Mutants expressing AG throughout the flower (arrowheads). (I) Anti-HA ChIP showing specific binding of AP2, TPL and HDA19 to the second AG intron. Control ChIPs were performed on non-transgenic wild-type tissue (Ler) or without antibody (no Ab). Data were normalized relative to input and ACT2 abundance. Data are represented as mean ± s.e.m. of at least two biological replicates. Student’s t-test was used to determine the significance of target enrichment relative to Ler IP (***P<0.005) or without antibody (no Ab). P<0.08). (J) Transgenes used in K,L. Domains are described in Fig. 1A. The TPL promoter is depicted as a black oval. (K) TPLp::TPL-AP2DBs rescues ap2-2 floral defects. Compare with A,B. (L) TPLp::AP2DBs is insufficient to rescue ap2-2. Scale bars: 1 mm in A-D; 50 μm in E-H.

**The AP2-TPL-HDA19 repressor complex negatively regulates the C-class gene AG**

We next sought to determine whether AP2 recruits TPL and HDA19 to regulate the C-class gene AG, which is expressed in whorls 3 and 4 and specifies the identities of the reproductive organs (Yanofsky et al., 1990). We performed RNA in situ hybridizations with TPL and HDA19 probes and found that both factors are broadly expressed in floral development and overlap with AP2 expression in outer whorl floral organ primordia, where AG is not normally expressed (supplementary material Fig. S2) (Wollmann et al., 2010). Additionally, genetic analyses showed that tpl-2 tpr1 tpr2 tpr3 tpr4 quintuple loss-of-function mutants and late-arising flowers of hda19-1 exhibit carpelloid outer whorl organs resembling those of strong ap2-2 mutants (Fig. 2B-D). All of these genetic backgrounds display AG misexpression throughout the flower (Fig. 2F-H) (Drews et al., 1991), indicating that AG is repressed not only by AP2, but also by redundantly acting members of the TPL/TPR family and HDA19. In the case of hda19-1, flowers begin to display carpelloid whorl 1 organs after ~20 flowers have been initiated. This is well before the termination of the inflorescence meristem, thereby distinguishing hda19-1 flowers from the ‘terminal carpelloid flower’ defects displayed by other mutants (Bao et al., 2004).

AP2 was recently shown to directly bind the second intron of AG (Yant et al., 2010; Dinh et al., 2012). Using chromatin immunoprecipitation (ChIP), we showed that this region of the second AG intron (supplementary material Fig. S3A) was specifically enriched in TPL and HDA19 immunoprecipitated

**Fig. 2. AP2, TPL and HDA19 cooperatively repress the C-class gene AG in outer whorl floral organs.** (A) Wild-type Arabidopsis flower composed of four sepals (se), four petals (p), six stamens (st) and two fused carpels (c). (B-D) Mutant flowers exhibiting carpel-like whorl 1 organs (arrowheads) with stigmatic tissue at their margins. Arrow in B designates stamen-like identity. (E-H) AG in situ hybridizations on stage 7-8 flowers. (E) Wild-type flower with expression restricted to stamen and carpel primordia. (F-H) Mutants expressing AG throughout the flower (arrowheads). (I) Anti-HA ChIP showing specific binding of AP2, TPL and HDA19 to the second AG intron. Control ChIPs were performed on non-transgenic wild-type tissue (Ler) or without antibody (no Ab). Data were normalized relative to input and ACT2 abundance. Data are represented as mean ± s.e.m. of at least two biological replicates. Student’s t-test was used to determine the significance of target enrichment relative to Ler IP (***P<0.005) or without antibody (no Ab). P<0.08). (J) Transgenes used in K,L. Domains are described in Fig. 1A. The TPL promoter is depicted as a black oval. (K) TPLp::TPL-AP2DBs rescues ap2-2 floral defects. Compare with A,B. (L) TPLp::AP2DBs is insufficient to rescue ap2-2. Scale bars: 1 mm in A-D; 50 μm in E-H.

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samples, as well as in AP2 control ChIP experiments (Fig. 2I; supplementary material Fig. S4A). Enrichment was diminished in ap2-2, a protein-null allele (Chen, 2004) but not absent (Fig. 2I), potentially owing to the ability of TPL to interact physically with redundantly functioning AP2 homologs (Causier et al., 2012). This decrease in enrichment was not due to a reduction of TPL or HDA19 levels (supplementary material Fig. S3D).

To test further the functional importance of AP2-mediated recruitment of TPL, we fused TPL to the DNA-binding domains of AP2 along with the recognition sequence of miR172 (which controls AP2 distribution) (Aukerman and Sakai, 2003; Chen, 2004). A transgene rescued ap2-2 floral defects (Fig. 2J,K), indicating that recruitment of TPL to AP2 target genes can functionally substitute for AP2. This also suggests that to correctly pattern perianth (sepal and petal) whorls, AP2 predominantly acts as a transcriptional repressor. In agreement with this, AP2 negatively regulated the expression of a reporter gene in perianth organs in a transcriptional repression assay (supplementary material Fig. S5). Collectively, our results indicate that AP2 functions as a transcriptional repressor in floral patterning, and does so by recruiting TPL and, in turn, HDA19. Moreover, this reveals a molecular mechanism for AP2-mediated repression of AG, as all complex members associate with the second intron of AG during floral development and repress its transcription in outer whorls.

**Fig. 3. AP2, TPL and HDA19 repress the B-class gene AP3 in whorl 1 organs. (A-D) Mutant Arabidopsis flowers with sepal-to-petal homeotic conversions (arrowheads). Compare with wild type in Fig. 2A. (E-H) Scanning electron micrographs of abaxial surfaces of floral organs. (E) Elongated epidermal cells of wild-type sepals are variable in size. (F) Slightly conical cells of wild-type petals are relatively uniform in size. (G) tpl-1 and (H) hda19-1 mutant whorl 1 organs with a sharp boundary between sepal and petal identity. (I-N) AP3 in situ hybridizations on stage 4 (I,J) and stage 7-8 (K-N) flowers. (I) AP3 is not expressed in young wild-type sepal primordia. (J) Ectopic AP3 expression in a young tpl-1 whorl 1 organ (arrowhead). (K) AP3 is detectable in later-stage wild-type sepals, but only at the organ base (arrows). (L-N) AP3 expression domains are expanded in mutant whorl 1 organs (arrowheads). (O) Anti-HA ChIP showing AP2, TPL and HDA19 specifically bind the AP3 promoter. Control ChIPs were performed on non-transgenic wild-type tissue (Ler) or without antibody (no Ab). Data were normalized relative to input and ACT2 abundance. Data are represented as mean ± s.e.m. of at least two biological replicates. Student’s t-test was used to determine the significance of target enrichment relative to Ler input (*P<0.05, **P<0.005) and the significance of decreased binding in ap2-2 (b, Pb<0.05). Scale bars: 1 mm in A-D; 100 µm in E-H; 50 µm in I-N.**

**AP2, TPL and HDA19 restrict petal fate by regulating the outer boundary of B-class gene expression**
Whereas tpl-2 tpr1 tpr2 tpr3 tpr4 quintuple mutant flowers resemble those of strong ap2 alleles, the tpl-1 mutant displays partial sepal-to-petal homeotic conversions (Fig. 3A,G). Such conversions are also found in tpl-2 tpr1 tpr3 tpr4 quadruple loss-of-function mutant flowers (supplementary material Fig. S6A) indicating that tpl-1 is not a complete dominant negative for the TPL/TPR family during floral patterning, as it is during embryogenesis (Long et al., 2006). Flowers of tpl-1 ap2-2/+ mutants show a much higher frequency of sepal-to-petal conversions compared with tpl-1 alone (Fig. 3B; supplementary material Table S2). Therefore, TPL and AP2 appear to cooperatively repress petal identity in whorl 1, revealing a new role for AP2 in floral patterning that is not predicted by the ABC model. HDA19 is also involved in this repression, as similar homeotic transformations occur in early-arising flowers of hda19-1 (Fig. 3C,H), and the frequency of these is greatly increased in tpl-1 hda19-1 mutants (Fig. 3D; supplementary material Table S2). Finally, like ap2-2/+ hda19-1/+ heterozygous mutations enhance the frequency of tpl-1 sepal-to-petal conversions (supplementary material Table S2).

The sepal-to-petal conversions displayed by the mutant combinations described above suggest misregulation of the B-class genes AP3 and PI in the first floral whorl. In wild type, both genes
are predominantly expressed in whorls 2 and 3 where they cooperatively specify petal and stamen fate (Fig. 3I,K; Fig. 4A,C) (Jack et al., 1992; Goto and Meyerowitz, 1994). AP3 is also expressed at the base of wild-type sepals in later stages of their development (Fig. 3K) (Weigel and Meyerowitz, 1993). PI expression is not detectable in whorl 1 organs at any stage (Goto and Meyerowitz, 1994). In flowers of tpl-1, ap2-2 and hda19-1 mutant combinations, however, PI is ectopically expressed in outer whorl organs (Fig. 4B,D-F), and AP3 is expressed precociously in newly initiated sepal primordia and in a much larger domain in older whorl 1 organs (Fig. 3J,L,N). The whorl 1 homeotic conversions displayed by these mutants not only correlate with this B-class gene misregulation but depend upon it, as either ap3-3 or pi-1 completely suppresses sepal-to-petal transformations (supplementary material Table S2).

To determine whether AP2 recruits TPL and HDA19 to directly repress B-class genes, we performed ChIP experiments on all three promoters. Consistent with published AP2 ChIP with high-throughput sequencing (ChIP-seq) data (Yant et al., 2010), we did not detect binding of AP2 to the PI promoter. However, AP2, TPL and HDA19 did bind a common promoter region of AP3 (Fig. 3O; supplementary material Fig. S3B, Fig. S4B), and binding by TPL and HDA19 was reduced in ap2-2 (Fig. 3O). Although AP3 was not reported as being significantly bound by AP2 in ChIP-seq analyses (Yant et al., 2010), evidence of AP2 binding to this region of the AP3 promoter could be detected in the raw data available from this study. Therefore, the role of AP2 in restriction of petal fate appears to involve the direct repression of AP3 and, probably, the indirect repression of PI.

Based on these regulatory relationships, ectopic B-class gene expression would be predicted in an ap2 mutant background. Indeed, AP3 and PI levels are variably misexpressed in whorl 1 organs of ap2-2 flowers (supplementary material Fig. S6B,C) (Goto and Meyerowitz, 1994). Consistent with this misexpression, ap2-2 carpelloid whorl 1 organs often produce tissue with stamen identity at their margins (Fig. 2B) (Bowman et al., 1991). By contrast, AP3 and PI levels are vastly reduced in their normal domains of expression in strong ap2 mutants (supplementary material Fig. S6B,C) (Jack et al., 1992; Goto and Meyerowitz, 1994). This apparent contradiction can be explained by the loss of whorl 2 and 3 organs in ap2 due to the spread of C-function, which plays a role in meristem determinacy (Jack et al., 1992; Goto and Meyerowitz, 1994). As a result, decreased expression of B-class genes might simply be an indirect consequence of reduced cellular proliferation within the floral whorls in which they act. In agreement with this, whorl 2 and 3 organ production and the expression domains of AP3 and PI are restored in an ap2 ag double mutant background (supplementary material Fig. S6E,F) (Zhao et al., 2007).
All modifications were increased to some extent in hda19-1 flowers compared with wild type, suggesting that HDA19 deacetylates these histone residues in planta (Fig. 6A). Because H4K16Ac showed the greatest increase, we performed ChIP using an antibody against this mark to assess acetylation levels at AP2 target loci in ap2-2 and wild-type flowers (Fig. 6B). Regulatory regions of both AG and SEP3 that were bound by AP2, TPL and HDA19 showed significantly increased H4K16Ac levels in ap2-2 (Fig. 6B), even though global levels of H4K16Ac in this background appeared comparable to wild type (Fig. 6A). These results are consistent with our model in which AP2 recruits a TPL-HDA19 repressor complex, as in the absence of AP2, histone marks controlled by HDA19 increase at AP2 target loci. These observations also suggest that unlike the global effect of hda19-1 on histone acetylation marks, the ap2-2 mutation affects H4K16Ac levels at specific loci. H4K16Ac abundance was only moderately increased at the AP3 promoter in ap2-2 (Fig. 6B), possibly due to the small fraction of cells that misexpress AP3 in the mutant flower.

DISCUSSION

In this study, we show that, in combination with TPL and HDA19, AP2 acts as a cadastral regulator of multiple floral homeotic genes to pattern the flower correctly (Fig. 7), broadening our knowledge of how gene expression boundaries are maintained in eukaryotic development. This includes the C-class gene AG, the outer expression boundary of which is controlled by an AP2-TPL-HDA19 repression complex. We therefore determine a molecular mechanism underlying a genetic relationship that contributed to the original formulation of the ABC model (the antagonism of AG by AP2).

We demonstrate further that AP2 recruits TPL and HDA19 to restrict whorl 1 expression of AP3, SEP3 and subsequently PI. This elucidates the presence of a repressor complex that establishes the peripheral expression boundaries of not only B-class genes but also the E-class gene SEP3, for which the restricted domain of activity in young flowers is unique among the SEPs (Mandel and Yanofsky, 1998). Our genetic analyses indicate that this regulation is an important aspect of floral patterning, as it is required to prevent the outward spread of petal fate. This represents a novel role for AP2 not predicted in the original ABC model, and in fact appears to disagree with the designation of AP2 as an A-class gene responsible for the specification of perianth fate (see below). Taking into account these new roles, AP2 has now been shown to directly restrict the expression domains of B-, C- and E-class genes in the flower (Fig. 7). Therefore, it is increasingly apparent that AP2 patterns the flower as a transcriptional repressor, a notion
strongly supported by the observation that expression of a fusion protein between the AP2 DNA-binding domains and a transcriptional co-repressor is sufficient to rescue ap2 floral patterning defects (Fig. 2K).

Although AP2, TPL and HDA19 are all expressed in whorls 1 and 2, petal fate is repressed only in the outermost whorl. The underlying cause of this positional difference is unknown, but it is plausible that AP2 constitutively represses SEP3 and B-class genes, but in whorl 2, antagonizing activators of petal fate, such as the F-box protein UNUSUAL FLORAL ORGANS (UFO), overcome this repression (Lee et al., 1997). UFO acts as a co-factor with the meristem identity gene LEAFY (LFY) to activate B-function (Lee et al., 1997). Whereas LFY is ubiquitously expressed in young flowers, UFO is transiently expressed in whorls 2 and 3, conferring domain specificity to LFY activity, which includes upregulation of target genes such as AP3, PI and SEP3 (Lee et al., 1997; Winter et al., 2011). Ectopically expressing UFO throughout the flower results in sepal-to-petal conversions and AP3 misexpression in whorl 1 organs (Lee et al., 1997), showing that UFO activity is epistatic to AP2-mediated repression of B-function. It is therefore not surprising that petal fate arises in whorl 2, where UFO is normally expressed, despite the presence of the AP2 repressor complex. Somewhat paradoxically, data indicate that UFO promotes transcription at the AP3 promoter by targeting LFY (or an associated factor) for degradation (Chae et al., 2008). In light of our results, UFO might function at the AP3 promoter to destabilize a repressor (Samach et al., 1999), such as AP2 and/or one of its complex members.

A balance between transcriptional activation and repression seems to underlie numerous aspects of floral organ fate specification. For example, activators of AG in the central domains of the flower compete with broadly distributed repressors of C-function to elicit region-specific transcription (Sridhar et al., 2006). This antagonism may ‘fine-tune’ AG expression levels to ensure appropriate floral organ identity. Such tuning mechanisms appear to exist in Antirrhinum and Petunia, in which mutations resulting in increased expression levels of C-class genes in their natural domains of expression result in the outward expansion of C-function (Cartolano et al., 2007).

The identification of an AP2-TPL-HDA19 repressor complex involved in the direct repression of AG reveals increased functional redundancy between transcriptional regulators known to inhibit C-function (reviewed by Kaufmann et al., 2010a). For example, LUG, a Gro/Tup1-type co-repressor structurally similar to TPL, and its interacting partner SEU appear to be recruited by the A-class regulator AP1 (and SEP3) to repress AG (Sridhar et al., 2006; Liu and Karmarkar, 2008). LUG has been shown to interact physically with HDA19 (Gonzalez et al., 2007), indicating that AP1-SEU-LUG and AP2-TPL repressor complexes might share chromatin-modifying factors to repress AG in outer whorl organs. Additionally, LUG and SEU might be recruited by AP2 to negatively regulate target genes not examined in the present work (Grigorova et al., 2011). These observations indicate that physical interactions between factors operating redundantly in the repression of AG may be elaborate.

The transcriptional regulation of the B-class gene AP3 also appears to be complex in nature. AP3 promoter analyses have identified cis-acting elements required for expression in early floral development (stage 3-5), along with elements necessary for later expression in petals and stamens (Hill et al., 1998; Tilly et al., 1998). Three MADS-domain binding sequences (CarG) reside in the AP3 promoter, the most proximal of which is apparently involved in the recruitment of a negative regulator affecting overall expression levels (Tilly et al., 1998). Promoter deletions, however, failed to identify elements potentially bound by AP2 or other repressors that restrict the outward spread of AP3 expression. The close proximity of our elucidated AP2-TPL-HDA19 binding site to activating CarG sites in the AP3 promoter (supplementary material Fig. S3B) might have complicated such analyses.

Although AP2 is clearly an important cadastral regulator of multiple floral organ identity genes, it is not obvious how AP2 acts as a floral organ identity gene itself (as described in the ABC model). Based on its classification as A-function, one of the proposed roles of AP2 is the specification of perianth fate. It is...
revealed in this study are in floral patterning events outside of Arabidopsis. SEP3 and B-class gene expression is commonly excluded from first whorl floral organs of angiosperms (Malcomber and Kellogg, 2005; Zahn et al., 2005a; Zahn et al., 2005b), and it will be of great interest to determine the role of AP2, TPL and HDA19 orthologs in this regulation. The perianths of some monocots and basal eudicots consist of two outer whorls of petaloid organs that express SEP3 or B-class genes (Kramer et al., 2003; Malcomber and Kellogg, 2005; Theissen and Melzer, 2007) and variations in AP2, TPL and HDA19 function in these species might contribute to this outward spread of petal fate. Characterizing such variation will provide valuable insight into the transcriptional mechanisms underlying the seemingly endless floral diversity of angiosperms.

Fig. 7. Model of AP2 function during flower development. AP2 controls the outer expression boundaries of B-, C- and E-class genes by recruiting TPL and HDA19. This AP2 repression complex directly represses the C-class gene AG in whorls 1 (sepal) and 2 (petal) and the B-class gene AP3 and E-class gene SEP3 in whorl 1. AP2-mediated repression of the B-class gene PI in whorl 1 may occur through negative regulation of upstream activators, such as SEP3. MADS-domain products of the floral homeotic genes (colored circles) are predicted to interact with one another and with SEP proteins to form quartet complexes that regulate gene expression required for organ identity specification (Honma and Goto, 2001). Intermediary proteins might facilitate TPL and HDA19 association.

difficult to reconcile how AP2 can play an instructive role in perianth fate while negatively regulating genes (B-class and SEP3) responsible for promoting petal identity. It is possible that AP2 represses petal fate in whorl 1 and promotes it in whorl 2. Indeed, the normal domain of B-class gene expression is reduced in ap2; however, previous reports attribute this reduction to organ loss resulting from C-function spread (Jack et al., 1992; Goto and Meyerowitz, 1994). The restoration of AP3 and PI expression in whorls 2 and 3 of ap2 ag shows that AP2 is not necessary for B-class gene activation (supplementary material Fig. S6E,F). These restored organs show a mixture of petal and stamen fate (Bowman et al., 1991), further indicating that AP2 is not strictly required for petal identity. If AP2 were required, the ABC model predicts that its expression would expand into the central region of indeterminate ag flowers (which display reiterations of perianth organs), but this does not occur (Wollmann et al., 2010). Together, these results indicate that AP2 does not directly specify perianth fate and appears not to fit its original A-function designation. This is in agreement with other reports suggesting that the disruption of perianth identity in ap2 mutants is an indirect effect reflecting an earlier role for AP2 in floral meristem specification (Okamura et al., 1997; Witt, 2007; Causier et al., 2010). In this context, AP2 first contributes to floral meristem identity (required for the production of the perianth) and later regulates the expression domains of B-, C- and E-class genes that specify floral organ fate. This interpretation does not exclude the possibility that other factors confer true A-function in Arabidopsis and other plant species.

A future challenge will be to determine how well conserved the novel protein interactions and transcriptional processes
Repression of floral organ genes


