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Discrete spatial and temporal *cis*-acting elements regulate transcription of the *Arabidopsis* floral homeotic gene *APETALA3*

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SUMMARY

The *APETALA3* floral homeotic gene is required for petal and stamen development in *Arabidopsis*. *APETALA3* transcripts are first detected in a meristematic region that will give rise to the petal and stamen primordia, and expression is maintained in this region during subsequent development of these organs. To dissect how the *APETALA3* gene is expressed in this spatially and temporally restricted domain, various *APETALA3* promoter fragments were fused to the *uidA* reporter gene encoding β-glucuronidase and assayed for the resulting patterns of expression in transgenic *Arabidopsis* plants. Based on these promoter analyses, we defined *cis*-acting elements required for distinct phases of *APETALA3* expression, as well as for petal-specific and stamen-specific expression. By crossing the petal-specific construct into different mutant backgrounds, we have shown that several floral genes, including *APETALA3, PISTILLATA, UNUSUAL FLORAL ORGANS,* and *APETALA1,* encode *trans*-acting factors required for second-whorl-specific *APETALA3* expression. We have also shown that the products of the *APETALA1, APETALA3, PISTILLATA* and *AGAMOUS* genes bind to several conserved sequence motifs within the *APETALA3* promoter. We present a model whereby spatially and temporally restricted *APETALA3* transcription is controlled via interactions between proteins binding to different domains of the *APETALA3* promoter.

Key words: *Arabidopsis*, *APETALA3*, Homeotic gene, Transcriptional regulation, Floral development

INTRODUCTION

The *Arabidopsis* flower consists of four whorls of organs: sepals, petals, stamens and carpels, which arise by proliferation of cells derived from the floral meristem. Genes required for specifying the floral pattern have been placed into three major classes: meristem identity genes, organ identity genes and cadastral genes (Jack et al., 1993). The meristem identity genes are necessary for proper establishment of the floral meristem. One role of these genes appears to be the activation of the organ identity genes, which in turn are required for appropriate organ development in their respective whorls. The cadastral genes are thought to control the expression boundaries of the organ identity genes.

Two organ identity genes, *APETALA3 (AP3)* and *PISTILLATA (PI)*, appear to be uniquely required for the development of petals and stamens. Mutations in either of these genes produce similar homeotic phenotypes and disrupt the specification of petal and stamen identity, such that second whorl organs develop as sepaloid structures, and the third whorl organs are carpeloid (Bowman et al., 1989; Hill and Lord, 1989). In wild-type plants, *AP3* transcripts are first detectable shortly after the first whorl sepal primordia begin to form at stage 3 (Smyth et al., 1990) in a meristematic domain that will give rise to the petals and stamens (Jack et al., 1992). As the petal and stamen primordia emerge, *AP3* RNA is present in all the cells of these organs at a fairly high level. *AP3* transcripts continue to be detected in both petals and stamens until the time of fertilization (Jack et al., 1992). In addition, *AP3* transcripts are detected late in floral development in the integuments of the developing ovules (Jack et al., 1992).

The regulation of *AP3* transcription can be divided into two stages: the initiation of expression and the maintenance of expression. Appropriate initiation of *AP3* expression is dependent on the activity of the meristem identity genes *LEAFY (LFY)* and *APETALA1 (AP1)*. *AP3* expression is reduced in strong *lfy* alleles, suggesting that *LFY* is a positive regulator of *AP3* transcription (Weigel and Meyerowitz, 1993). The effects of *LFY* on *AP3* transcription may be mediated in part by the action of the *UNUSUAL FLORAL ORGANS (UFO)* gene product, since *UFO* appears to act in concert with *LFY*, and *ufo* mutants show a decrease in the levels of *AP3* expression (Levin and Meyerowitz, 1995; Lee et al., 1997). In *ap1* mutant flowers, *AP3* is expressed essentially as in wild type (Weigel and Meyerowitz, 1993). In *lfy ap1* double mutants, however, *AP3* transcripts are generally non-detectable at all stages of development, suggesting that *AP1* in combination with the *LFY* pathway positively regulates *AP3* expression (Weigel and Meyerowitz, 1993). However, these gene products alone do not appear to be sufficient for spatially

limiting the activation of *AP3* to just the petal and stamen primordia.

The maintenance of *AP3* expression in the petal and stamen primordia depends on both auto-and cross-regulatory interactions. *SUPERMAN* (*SUP*) is classified as a cadastral gene, since flowers mutant for *sup* display ectopic stamens in the fourth whorl, and concomitant ectopic AP3 protein (Schultz et al., 1991; Bowman et al., 1992; Sakai et al., 1995). *AP3* transcription is initiated normally in *sup* mutant flowers, suggesting that *SUP* is required to maintain and delimit the boundary of *AP3* transcription once it is initiated (Sakai et al., 1995). *PI* is also required for the maintenance of *AP3* expression in its normal domain (Jack et al., 1992; Samach et al., 1997). The maintenance of *AP3* expression also depends on autoregulation. In the *ap3-3* nonsense mutant, early *AP3* expression is normal, but by stage 6 when the petal and stamen primordia have emerged, *AP3* RNA is undetectable (Jack et al., 1994). That *AP3* positively autoregulates its own expression has also been shown by overexpression of *AP3* under the control of the broadly expressed cauliflower mosaic virus (CaMV) 35S promoter which results in a partial conversion of the fourth whorl carpels to stamens, and concomitant activation of an *AP3* promoter-reporter gene fusion in the fourth whorl (Jack et al., 1994). However, the *AP3* and *PI* gene products alone are not sufficient to *trans*-activate expression of an *AP3* promoter-reporter gene fusion outside the flower, indicating that other factors must play a role in the transcriptional regulation of *AP3* (Krizek and Meyerowitz, 1996).

At least some of these effects on *AP3* transcription may be mediated by direct binding of these regulatory gene products to the *AP3* promoter. The deduced products of the *AP1, AP3*, *PI* and *AGAMOUS (AG)* floral homeotic genes all contain a MADS-domain, which is found in a number of transcription factors (Yanofsky et al., 1990; Jack et al., 1992; Mandel et al., 1992; Goto and Meyerowitz, 1994). Several MADS-domain containing proteins have been demonstrated to bind DNA at a core consensus binding site $CC(A/T)_6GG$ which is referred to as the CArG box (Schwarz-Sommer et al., 1992; Wynne and Treisman, 1992; Huang et al., 1993; Shiraishi et al., 1993). The *AP3* promoter region contains several CArG box motifs which may serve as binding sites for one or more MADS-domain containing proteins (Irish and Yamamoto, 1995). In vitro DNA binding studies have demonstrated that AP1 homodimers, AG homodimers, and heterodimers composed of AP3 and PI proteins can all bind to a synthetic oligonucleotide containing a CArG box sequence found in the *AP3* promoter, albeit with different affinities (Riechmann et al., 1996).

The products of the *SUP*, *LFY*, and *UFO* genes may also regulate *AP3* transcription directly. *SUP* encodes a putative protein containing a zinc finger and a leucine zipper, and thus may act as a transcription factor (Sakai et al., 1995). The biochemical function of the *LFY* gene product is not known, although *LFY* encodes a nuclear protein and has been postulated to act as a transcription factor (Weigel et al., 1992; Levin and Meyerowitz, 1995). A biochemical function for UFO is still unclear, although recent studies have suggested a role in cell proliferation and growth control (Ingram et al., 1995; Bai et al., 1996; Ingram et al., 1997).

These studies indicate that a number of gene products are required for appropriate *AP3* expression. Here we have defined *cis*-acting elements in the *AP3* promoter as well as identifying some of the potential *trans*-acting factors that may bind to these elements. Previously we have shown that the 1.7 kb region immediately upstream of the *AP3* coding region contains all of the essential *cis*-regulatory elements, since it is sufficient to rescue the *ap3* mutant phenotype when fused to an *AP3* cDNA (Irish and Yamamoto, 1995). We have fused different regions of this 1.7 kb *AP3* promoter to the reporter gene *uidA* encoding β-glucuronidase (GUS) (Jefferson et al., 1987), and assayed the pattern of expression produced by these constructs in transgenic *Arabidopsis* plants. Our results show that discrete domains of the *AP3* promoter confer tissue-specific patterns of expression; other domains appear to be required for appropriate temporal control of *AP3* transcription. The expression pattern conferred by the petal-specific element in various mutant backgrounds suggests that several floral genes are responsible for mediating transcriptional activation from this promoter fragment. Furthermore, we show that the AG, AP1, and AP3/PI proteins bind to multiple sequences within the *AP3* promoter. Based on these results we suggest a model whereby different aspects of *AP3* expression are regulated via interactions between *trans*-acting factors that bind to specific elements in the *AP3* promoter.

MATERIALS AND METHODS

DNA constructs

Fig. 2 shows the *AP3* 5′ region with the restriction sites used to create the promoter constructs for this study. All 5′ deletion constructs were derived from the 1.9 kb *Hin*dIII genomic fragment containing 1.7 kb of *AP3* 5′ sequence and the first 159 bp of *AP3* coding sequence. The 5′ deletion intermediates were created using the restriction sites *Eco*RV at position −927 (p5D3), *Ava*II at −727 (p5D4), *Ase*I at −556 (p5D5), *Sac*I at −328 (p5D6), *Ava*II at −285 (p5D6.5), *Cla*I at −227 (p5D7), and *Ava*II at −83 (p5D8). An engineered *Xba*I site directly 3′ to the ATG was created by PCR, and the ATG of each of the *AP3* promoter 5′ deletion constructs was replaced with this PCR product.

Internal deletion constructs were created by fusing selected 3′ and 5′ deletion fragments. The 3′ deletions were derived from pV51, which contains the same *AP3* genomic insert as pV15 except in the opposite orientation (Irish and Yamamoto, 1995). The pV51 plasmid was cut with *Sal*I and *Kpn*I, digested with ExoIII nuclease, and religated. The resulting plasmids were sequenced to determine the exact positions of the new 3′ end of the promoter fragments. These promoter fragments were combined with the deletion derivatives described above to generate the internal deletion constructs. The plasmids are named according to the respective ends of the internal deletions: for pDxy and pD3-xy, the x represents the 3′ deletion and the y identifies the 5′ deletion end (see Fig. 2). Details of individual plasmid constructions are available on request.

In frame translational fusions at the ATG of the *AP3* promoter deletion derivatives and the GUS reporter gene were created by cloning the various promoter constructs into the *Sal*I and *Bam*HI sites of pBI101 (Clonetech, Palo Alto, CA). The D6-DTA construct was created by replacing the 1.7 kb of *AP3* 5′ sequence in the pBIADN plasmid (Day et al., 1995) with the 5D6 deletion fragment. All binary vector expression constructs were transferred to *Agrobacterium tumefaciens* strain GV3101 by electroporation.

The *BobAP3* gene has previously been identified (Carr and Irish, 1997). Sequencing of the genomic *BobAP3* clone and verification of the sequence of promoter constructs was carried out using Sequenase 2.0 (United States Biochemical, Cleveland, OH). Numbering of bases in the *AP3* promoter are relative to the start of transcription as defined by Jack and colleagues (1992). Sequence comparisons were made

using GeneWorks 2.1 (Intelligenetics, Inc., Mountain View, CA). Enzymes used for subcloning were obtained from New England Biolabs, Beverly, MA.

Plant transformation and histochemical analysis

Plants were grown under 16 hour day/8 hour night conditions. Promoter-GUS constructs were introduced into the *Arabidopsis* genome (ecotype Landsberg *erecta*) via *Agrobacterium*-mediated transformation (Valvekens et al., 1988; Bechtold et al., 1993). Transformed lines were identified by kanamycin selection. Those lines giving 1 or 2 bands on a Southern blot were selected for further analysis.

Inflorescences taken from several progeny of at least 3 independent transgenic lines for each construct were stained for GUS enzyme activity in 2.5 mg/ml X-glucuronidase, 100 mM phosphate buffer, 1.0 mM EDTA, 10% Tween-20, and 0.0 to 5.0 mM potassium ferri/ferrocyanide for 18-36 hours. Tissues were then cleared in 70% ethanol, mounted in 50% glycerol and photographed on a Zeiss Axiophot microscope equipped with bright-field, dark-field and differential interference contrast optics. Images were assembled using Adobe Photoshop (Adobe Systems, Mountain View, CA).

DNA binding assays

Proteins for gel shift assays were produced by in vitro transcription of the *AP1*, *AP3*, *PI*, and *AG-ATG34* cDNAs (Mandel et al., 1992; Mizukami and Ma, 1992; Goto and Meyerowitz, 1994; Irish and Yamamoto, 1995) which were then translated in vitro using the Rabbit Reticulocyte Lysate System (Promega, Madison, WI). For AP3/PI binding studies, the *AP3* and *PI* mRNAs were co-translated. DNA probes were prepared by cutting various promoter constructs with the appropriate restriction enzymes then labeling the DNA by end filling with Klenow in the presence of [^{32}P]dCTP. The labeled DNA fragments were then purified from a non denaturing polyacrylamide gel. Binding reactions and gel conditions were as described (Schwarz-Sommer et al., 1992) except binding reactions contained 0.25-0.50 fmol labeled probe as well as 200 ng of each of the nonspecific carriers, poly(dI-dC) and calf thymus DNA. The following CArG box oligos were annealed with their complementary oligos and used for the competition assays: CArG 1, 5′- AAA TCA GTT T**AC ATA AAT GG**A AAA TTT ATC ACT-3′; CArG2, 5′-AAC TTC TGA ACT TA**C CTT TCA TGG** ATT AGG CAA-3′; CArG3, 5′-GCA ATA CTT T**CC ATT TTT AGT** AAC TCA AGT GGA-3′ (the putative CArG elements are in boldface).

RESULTS

Identification of putative regulatory domains within the *AP3* promoter by sequence similarity

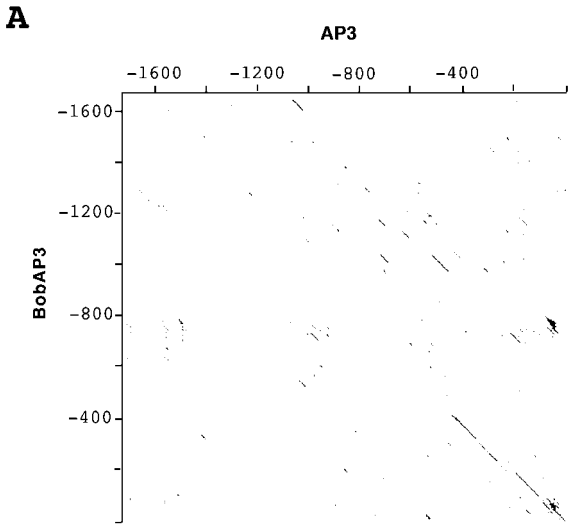
In order to define the domains within the 1.7 kb *AP3* promoter which might be important for transcriptional regulation, we isolated the upstream promoter region from the *AP3* homolog *BobAP3* from *Brassica oleracea botrytis* (cauliflower) (Carr and Irish, 1997). Sequence similarity between the *AP3* and *BobAP3* promoters is high (approximately 62% identity) over the region from −440 bp to the start of transcription (Fig. 1A). Further upstream, the similarity between the two promoters falls off significantly, although there are short blocks of sequence similarity beyond −440 bp. These conserved sequences represent candidate *cis*-acting regulatory elements. An open reading frame (ORF) contained within the 1.7 kb fragment is identical to an *Arabidopsis* EST (GenBank accession number Z33724) and similar to a maize triose phosphate/phosphate translocator protein (Fischer et al., 1994) (Fig. 2). Of the three CArG boxes which are found in the region of similarity, only CArG box 2 is completely conserved between the two species (Fig. 1B). The sequence of CArG box 3 is identical to a binding site for the AG floral homeotic gene product defined in vitro (Huang et al., 1993; Shiraishi et al., 1993).

Discrete *AP3 cis*-regulatory elements

In order to localize the regulatory elements of the *AP3* promoter, deletion derivatives of the 1.7 kb region were generated and in-frame translational fusions to the *E. coli uidA* gene which encodes the β-glucuronidase (GUS) enzyme were made (Fig. 2). A series of 5′ deletions were used to define the minimal region required for a wild-type pattern of expression. To more precisely localize specific regulatory regions of the promoter, a series of internal deletions were also made. These constructs were introduced into the *Arabidopsis* genome, and for each construct at least three independent stably transformed lines containing one or two inserts each were analyzed. Lines containing many inserts had variable patterns of GUS staining, and so were not used in this study (data not shown). Flowers from each line were stained for GUS enzyme activity and whole mounts of plant tissues were analysed for GUS localization (Table 1). The distribution of GUS enzymatic activity for each construct was similar for the different lines tested. We characterized expression patterns ‘early’ (stages 3-5, staged according to Smyth et al., 1990) and during the later stages of floral organ development.

Plants harboring the pAP3 construct containing the 1.7 kb *AP3* promoter displayed GUS expression in the second whorl petals and third whorl stamens. The transformants containing the 5D2, 5D3 and 5D4 constructs also showed a similar expression pattern in the flower (Fig. 3A-H, and data not shown). GUS expression in these lines was first faintly detectable at stage 3, in a ‘C’ shape, such that there was a gap on the adaxial side of the ring of expression (Fig. 3B,C). Slightly later during stage 3 these transgenic lines showed a complete ring of GUS expression throughout the domain that will give rise to the petals and stamens (Fig. 3C). Expression was maintained in this region through stage 6, when the petal primordia are still small and the stamen primordia are distinct (Fig. 3D,E). Low levels of staining were also seen on the adaxial side at the base of the sepals (Fig. 3D). Subsequent to stage 6, GUS expression was intense in the petal primordia, and also detected in the stamen primordia, but at a somewhat lower level than that seen in the petals. As the stamens began to elongate during stages 7-9, GUS expression was seen predominantly in the filaments and connectives. By stages 9-10, the levels of GUS expression became reduced in the petals and stamens (Fig. 3F). At anthesis, GUS activity was undetectable in the mature petals, but could still be detected in the connectives of the stamen (Fig. 3G). GUS activity was also seen in the funiculus and integument cells of the ovule (Fig. 3H). The patterns of GUS expression seen in these transgenic lines were similar to the patterns of *AP3* transcript accumulation during floral development (Jack et al., 1992; Weigel and Meyerowitz, 1993). Therefore, the minimum region necessary to confer a wild-type *AP3* expression pattern is contained within the 727 bp fragment directly upstream of the start of transcription.

Plants containing the 5D5 construct displayed a pattern of GUS expression that was quite similar to that conferred by the



B

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AP3      ---------- ---------- -----CTTTA GGGTAAGTGC AAAAAGCCAA  -417
BobAP3   TAGCGCCCAG GCTTCCCGAA CAGGGCTTTG GGGTAAG-GA AAAAGGCTAA

AP3      CCAAAT-CCA CCTGCACTGA TTTGACGTTT ACAAACGCCG TTAAGTTTGT  -368
BobAP3   CCAAATCCCA CCTGCAC--A CTTGACGTTT ACAAACGCCG TTAAGTATGT

AP3      CACCGTCTAA ACAAAAACAA AGTAGAAGCT AACGGAGCTC CGTTAATAAA  -318
BobAP3   CATCGTGTAA ACAAA----- -----AAGC- AA-------- -GT-------

AP3      TTGACGAAAA GCAAACCAAG TTTTTAGCTT TGGTCCCCCT CTTTTACCAA  -268
BobAP3   -----G---- GGAAACCAA- --CGAAGCTC CG--TGAATT AATAAACGGA

AP3      GTGACAATTG AT--TTAAGC AGTGTCTTGT AATTATACAA CCATCGAT-G  -221
BobAP3   AAG-CACACG ATGCCCATTT ATAGCTTTG- GTCCACTCTT CCACCTATAG

AP3      TCCGTTGA-T TTAAACAGTG TCTTGT-AAT TAA-----AA AAAT---CAG  -181
BobAP3   ACAATTGAGT TTAAGCAGTG TCTTGTAAAT TAACCATCAT ACATGTCCAT

                CArG1
AP3      T----TTACA TAAATGGAAA ATTTATCACT TAGTTTTCAT CAACTTCTGA  -135
BobAP3   TCCACTTACC AAAAAGGAAA GTTTATCACT TAGTTTTCAT CAACTTCTGA

              CArG2                          CArG3
AP3      ACTTACCTTT CATGGATTAG GCAATACTTT CCATTTTTAG TAACTCAAGT  -85
BobAP3   ACTTACCTTT CATGGATTAG GCAACACCTT TT-TATATAG TAAGTCTAGT

AP3      GGACCCTTTA CTTCTTCAAC TCCATCTCTC TCTTTCTATT TCACTTCT-T  -36
BobAP3   GGGTCCTTTG CTT------- TC--TTTTTC T-TTTCTCTT TCACTTCTCT

                                                  ▼
AP3      TCTTCTCATT ATA-TCTC-T TGTCCTCTCC ACCAAATCTC TTCAACAAAA  +13
BobAP3   TCTTCTTATT ATAATCTCTT TGTCCTCTTC GCCAAATCTC TTCAACAAAA

AP3      -AGATTAAAC --AA-AGAGA GAAGAAT-AT G                      +39
BobAP3   TATCAAAAAC TAAATAGAGA GAAGAATAAT G
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Fig. 1. Sequence similarities between the *AP3* and *BobAP3* promoters. (A) Sequences 5′ to the presumed initiation codons of the *Arabidopsis AP3* gene (Jack et al., 1992; Irish and Yamamoto, 1995) and the homologous *Brassica oleracea botrytis BobAP3* gene are compared in a dot matrix alignment. 1760 nucleotides of the *AP3* sequence is shown on the X axis, and 1674 nucleotides of the *BobAP3* sequence is on the Y axis. The comparison shows sequences with a similarity of greater than 50% over a range of 25 nucleotides. (B) Gapped alignment of the 440 bp region upstream of the start of translation of *AP3* as compared to the homologous region of the *Bob AP3* gene. Regions of sequence identity are indicated by dots, and the three CArG boxes contained within this region of the *AP3* promoter are shown in bold. Only the CArG box 2 sequence is completely conserved between *Arabidopsis* and *B. oleracea botrytis*. The transcription initiation site at position +1 of the *AP3* gene (Jack et al., 1992) is marked with an arrowhead. A putative TATA box is located 28 bp upstream of the *AP3* transcription start site. The GenBank accession number for the *BobAP3* genomic sequence is AF043610.

longer promoter constructs (Fig. 3J). However, only low levels of GUS expression were detected up until stage 6 (Fig. 3I). The difference in staining intensities seen between the 5D4 and 5D5 lines suggests that the promoter region from −727 to −556 is required to enhance expression from stages 3 to 5.

Transformants containing the 5D6 and 5D6.5 constructs showed weak GUS activity early at stages 3 to 5, and in the developing petal primordia at stages 6 and later (Fig. 3K,L). GUS expression was maintained in this petal-specific domain throughout subsequent stages of development until anthesis. GUS enzyme activity was not detectable at any stage in the developing stamens in the 5D6 or 5D6.5 lines. A very weak petal-specific pattern of expression commencing at about stage 6 was seen in the 5D7 transgenic lines (Table 1). No GUS activity was detected in the flowers of transgenic lines containing the 5D8 construct. The 5D8 construct contains only 83 bp of promoter sequence, and so lacks the three CArG boxes.

To test the tissue specificity of the petal-specific promoter fragment, we generated a translational fusion of the 328 bp promoter fragment present in 5D6 to the diphtheria toxin A chain (DTA) gene, and introduced this construct into *Arabidopsis* plants. DTA gene expression results in cell ablation, and has been used as a reporter gene in *Arabidopsis* (Thorsness et al., 1993; Day et al., 1995). Flowers from transgenic plants containing a single copy of the 5D6-DTA construct completely lacked petals, but the other floral organs appeared phenotypically normal (Fig. 3S,T). In a few lines with multiple inserts, we observed occasional loss of stamens in the more acropetal flowers, indicating that this promoter element can confer a low level of expression in the third whorl. Despite the fact that the 5D6 promoter construct confers low levels of GUS expression in the ovules, the 5D6-DTA transgenic plants were fertile. This may be due to the fact that low levels of DTA expression have been correlated with lack of ablation (Day et al., 1995).

A series of internal deletions were used to further delimit the *cis* elements required for various aspects of expression. Internal deletions were generated in the context of the pAP3 or 5D3 promoter constructs. Since it appeared that sequences between −727 and −328 enhanced early (stage 3-5) expression and were required for stamen-specific expression, we generated several constructs which had internal deletions lacking parts of this region. Transgenic flowers harboring the D36 or D3-36 constructs which contain an internal deletion from −615 to −328 still displayed strong GUS expression early in development, comparable to the levels seen with the 5D4 construct (Fig. 3M). Expression in the developing stamen primordia was low in these transgenic flowers until approximately stage 12, when expression could be detected in the filaments and connectives (Fig. 3N and data not shown). Plants containing the D26 or D3-26 constructs, though,

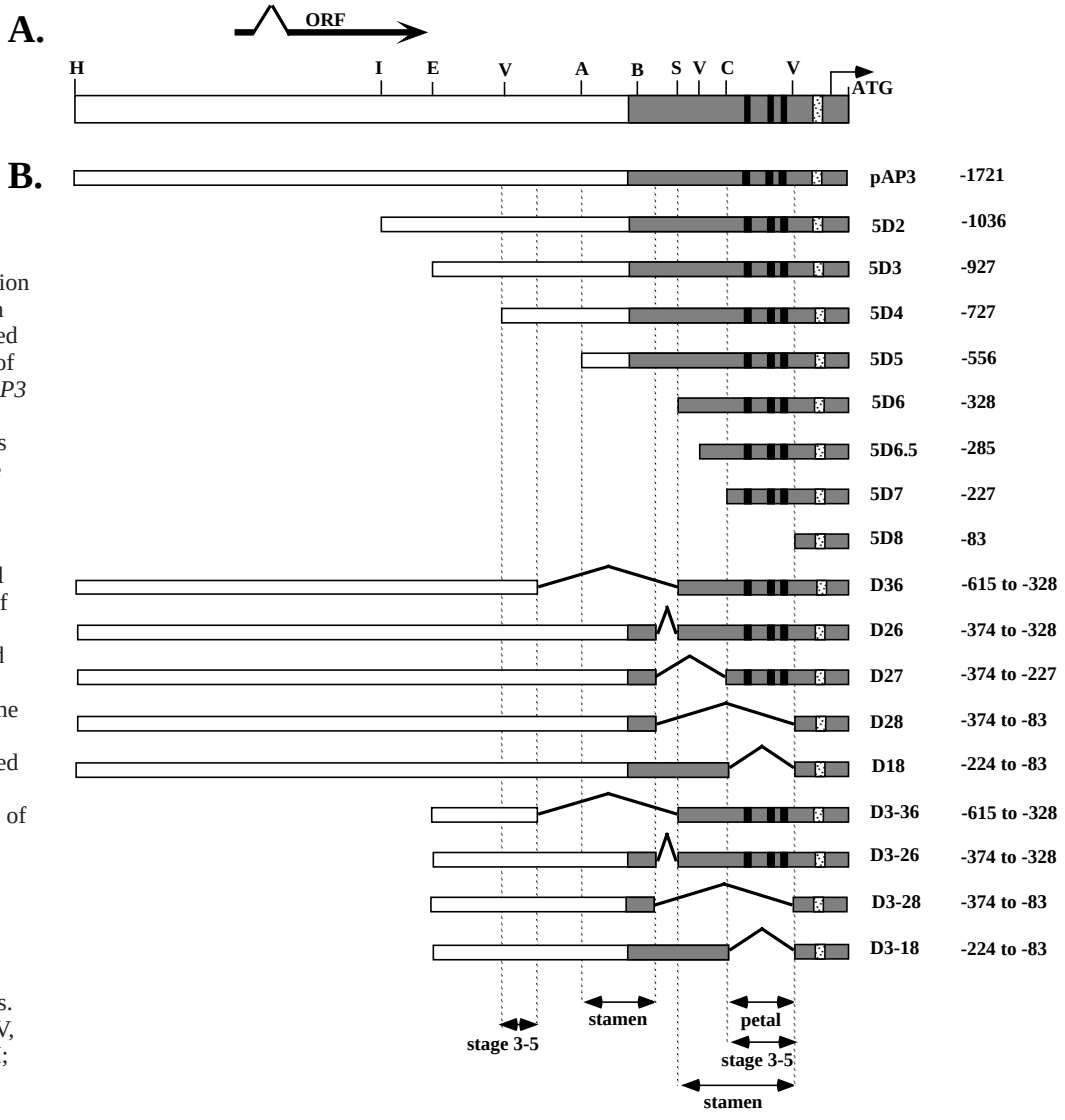

Fig. 2. *AP3* promoter constructs. (A) The 1.7 kb *AP3* promoter region is shown, with selected restriction enzyme sites indicated. The shaded region corresponds to the region of high similarity between the *BobAP3* and *AP3* promoter regions. The positions of the three CArG boxes within the region of similarity are denoted in black. The putative TATA element is marked by a stippled region, and the start of transcription is marked by a small arrow. The ATG marks the start of translation. The approximate position of the upstream ORF and its direction of transcription is shown above the promoter. (B) The *AP3* promoter regions used in the GUS fusion constructs are depicted schematically. The lengths of the promoter elements, or the lengths of the internal deletions, are listed. Based on the analyses of GUS staining patterns (see text), the positions of putative *cis*-acting elements required for different aspects of expression are shown relative to the promoter constructs. H, *Hin*dIII; I, *Hin*cII; E, *Eco*RV; V, *Ava*II; A, *Ase*I; B, *Bsp*MI; S, *Sac*I; C, *Cla*I.

Table 1. Summary of expression patterns of AP3 promoter-GUS fusions

Construct	Stages 3-5 early expression	Stage 6 petal primordia	Stage 6 stamen primordia	Stage 10-12 petals	Stage 10-12 filaments	Stage 10-12 anthers	Ovules
5D2	+++	+++	++	++	+++	+	++
5D3	+++	+++	++	++	+++	+	++
5D4	+++	+++	++	++	+++	+	++
5D5	+	+++	++	+	++	+	+
5D6	+	+++	–	+	–	–	+
5D6.5	+	+++	–	+	–	–	–
5D7	–	+	–	–	–	–	–
5D8	–	–	–	–	–	–	–
D36	+++	+++	–	++	+*	–	++
D26	+++	+++	++	+	++	+	++
D27	+++	+++	–	++	+*	–	–
D28	–	–	–	–	–	+	–
D18	–	–	–	–	–	+	–
D3-36	+++	+++	–	++	+*	–	++
D3-26	+++	+++	++	++	+++	+	++
D3-28	–	–	–	–	–	+	–
D3-18	–	–	–	–	–	+	–

Relative levels of GUS staining denoted by: +++, high; ++, moderate; +, low; –, no staining detectable.
*in connectives only.

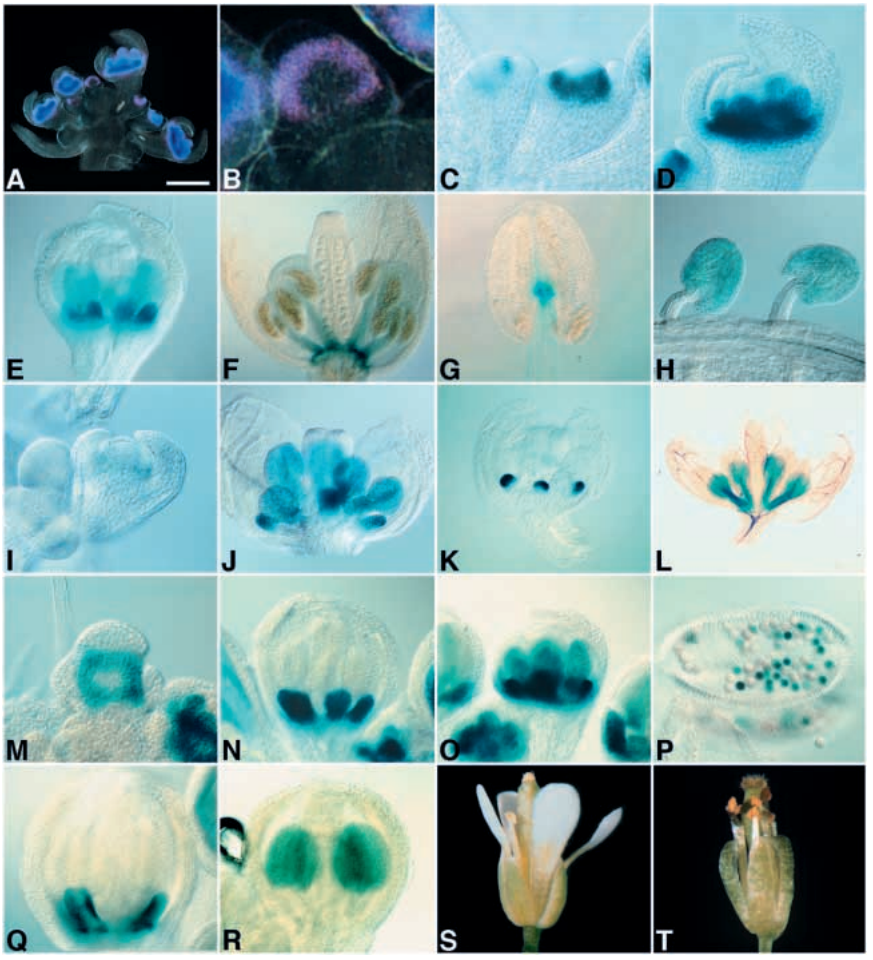

Fig. 3. Expression patterns conferred by AP3 promoter constructs. (A-H) GUS expression conferred by the 5D3 construct. (A) Dark-field view of an inflorescence showing expression limited to developing flowers. (B) Dark-field top view of an early stage 3 flower with GUS expression in a 'C' shape. (C) Early and late stage 3 flower buds; (D) late stage 5; (E) stage 6; (F) stage 10 flower buds. (G) GUS staining is seen predominantly in the connectives of the mature anther. (H) GUS staining is also seen in ovules. (I,J) GUS expression conferred by the D5 construct at (I) stage 3 and (J) stage 7. (K,L) GUS expression conferred by D6 construct at (K) stage 6 and (L) stage 10. (M,N) GUS expression conferred by D3-36 construct at (M) stage 3, (N) stage 7. (O) GUS expression at stage 6 in transgenic D3-26 flower. (P) GUS expression is segregating in the pollen from a transgenic D28 plant. (Q) GUS expression in the developing petals of a stage 7 transgenic D27 flower. (R) GUS expression in the anthers of a transgenic D3-18 plant. (S, T) phenotypes of (S) wild type as compared to (T) transgenic D6-DTA flower lacking petals. Scale bar (A,F) 200 μm; (G) 150 μm; (E,H,J,K,N,O,P,Q,R) 100 μm; (C,D,I,M) 50 μm; B, 40 μm; L, 400 μm.

retained expression in the developing stamen primordia (Fig. 3O). These observations suggest that the region from −727 to −615 is required to enhance expression from stages 3 to 5, while the sequences from −556 to −374 are required to enhance expression in developing stamen primordia (Fig. 2).

To further define the sequences necessary for other aspects of expression, we generated constructs which had deletions of sequences between −374 and −83. Deletion of this entire region in the context of the full length promoter (constructs D3-28 and D28) resulted in weak GUS activity in the anthers late in development (stages 10 until anthesis) and in the pollen (Fig. 3P). A similar pattern of expression was conferred by the D3-18 and D18 constructs, which were deleted for sequences from −224 to −83 (Fig. 3R). However, D27, which is deleted for sequences from −374 to −227 retains early and petal-specific expression, and lacks expression in the developing stamen primordia (Fig. 3Q). Since the D3-26 and D26 lines retained all aspects of the wild-type expression pattern, it appears that the sequences from −374 to −328 are not required to mediate expression, and that stamen-specific *cis*-acting sequences can be subdivided into two domains, from −555 to −374, and from −328 to −83. These results taken together suggest that the sequences from −224 to −83 are required for early (stages 3-5) and for petal-specific expression during floral development. Sequences from −555 to −374 appear to be required for anther-specific expression in the stamens, while the region from −328 to −83 is required for expression in the filaments. The presence of both stamen-specific elements appears to be necessary to elicit the entire spectrum of stamen-specific expression.

Floral homeotic genes mediate specific aspects of *AP3* expression

The 5D6 construct lacks one of the stamen-specific elements and one of the early elements that we mapped by deletion analysis (Fig. 2). The 5D6 construct confers a low level of expression early, at stages 3-5, and second whorl petal-specific expression at stages 6 and later (Fig. 4A). Therefore, since the 5D6 construct is lacking some of the redundant elements required to confer the full spectrum of *AP3* expression, we used it to test which floral homeotic genes are required to mediate expression from a subset of the *cis*-acting elements. We crossed the 5D6 construct into a number of mutant backgrounds, the resulting plants were self-fertilized, and progeny homozygous for a given mutation were analyzed.

We assayed the GUS expression pattern produced by the 5D6 construct in plants mutant for either the strong *ufo-2* allele, or the strong *ap1-1* allele. Mutations in *UFO* result in more inflorescence-like flowers that contain variable homeotic transformations of the floral organs (Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). We could not detect

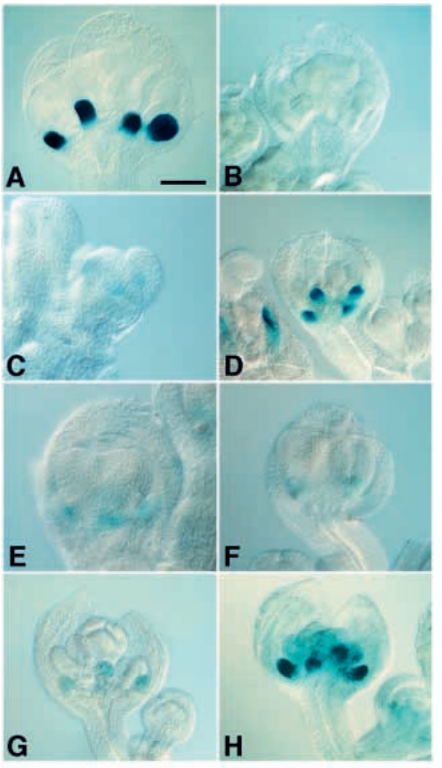

Fig. 4. GUS expression conferred by the 5D6 construct in various mutants. (A) Wild type, (B) *ufo-2*, (C) *ap1-1*, (D) *sup*, (E) *ap3-4*, (F) *pi-1*, (G,H) *ag-1*. Scale bar, 100 μm.

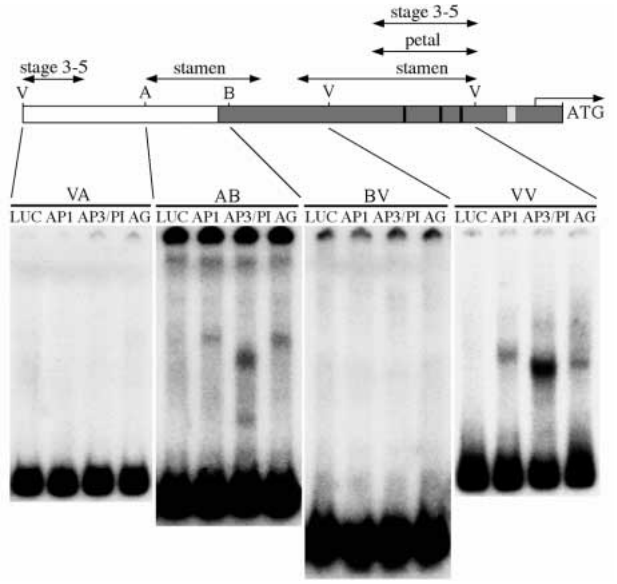

Fig. 5. AP1, AP3/PI, and AG binding to specific regions of the *AP3* promoter. The region of the *AP3* promoter required for transcriptional regulation is illustrated at the top, with notation as in Fig. 2. The fragments used as probes are indicated as VV (*Ava*II-*Ava*II), BV (*Bsp*MI-*Ava*II), AB (*Ase*I-*Bsp*MI), VA (*Ava*II-*Ase*I). Below are shown EMSA of *AP1*, *AP3/PI*, or *AG* reticulocyte lysate translation products with the four *AP3* promoter probes. The Luciferase (LUC) control translation reaction was used as a nonspecific binding control.

either early or second whorl-specific 5D6 transgene expression in *ufo-2* flowers (Fig. 4B). Occasionally, *ufo-2* plants produce flowers with some petals or stamens; in a few cases, we observed GUS staining in the developing petals of such *ufo-2* flowers (data not shown). Mutations in *AP1* also result in flowers which have a more inflorescence-like character, and in addition lack petals (Irish and Sussex, 1990; Bowman et al., 1993). In contrast to the lack of 5D6 expression in *ufo* mutant flowers, we find weak early 5D6 expression in the *ap1-1* mutant background, but do not detect later, second whorl-specific expression (Fig. 4C).

SUP has been postulated to act to maintain the boundary of *AP3* expression (Sakai et al., 1995). There are two possibilities for the expression of the 5D6 transgene in a *sup* mutant background. If *SUP* acts by delimiting third whorl *AP3* expression, then we should see no effect on 5D6 expression in a *sup* mutant. Alternatively, *SUP* may act to regulate the overall size of the *AP3* expression domain, and so we might expect to see ectopic 5D6 transgene expression in *sup* mutant plants. In keeping with the first alternative, we find that 5D6 expression is normal early, and restricted to the developing petal primordia at stages 6 and later in *sup-1* mutant floral buds (Fig. 4D).

Both *AP3* and *PI* are required for the maintenance of *AP3* transcription at stages 6 and later (Jack et al., 1992). We find that the 5D6 transgene is expressed at low levels at stage 5 in both *pi-1* and *ap3-4* mutant flowers, however no expression can be detected at later stages (Fig. 4E,F).

Strong *ag* mutations result in a homeotic conversion of stamens into petals as well as an indeterminate growth pattern, and show no alteration in *AP3* expression (Bowman et al., 1989; Jack et al., 1992). We crossed the 5D6 transgene into an *ag-1* mutant background, and examined the resulting GUS expression patterns. Despite the fact that *ag-1* mutant plants display a homeotic transformation of third whorl organs into petals, we observed GUS expression only in the second whorl organs of stage 6 *ag-1* floral buds (Fig. 4G). Slightly later, as GUS expression becomes more intense in the second whorl organs, GUS expression also becomes apparent in the third whorl significantly after the third whorl primordia have arisen and begun to develop (Fig. 4H). GUS expression in the third whorl remains low and never becomes as intense as that seen in the second whorl organs.

AP3, PI, AP1 and *AG* bind to several AP3 promoter sequences

We assayed the ability of candidate *trans*-acting protein factors to bind to sequences within the −727 to −83 region of the *AP3* promoter. We focussed on the MADS box containing AP1, AP3/PI and AG proteins as potential *trans*-acting factors, since they have overlapping expression patterns with *AP3*, and there are several putative CArG box elements within the *AP3* promoter. This 644 bp promoter region was divided into four domains, and each of these domains was tested for its ability to bind in vitro synthesized AP1, AP3/PI, and AG using electrophoretic mobility shift assays (EMSA).

When translated and used alone, neither the AP3 nor the PI protein was able to show DNA binding to any of the sequences contained within the 644 bp region (data not shown). However,

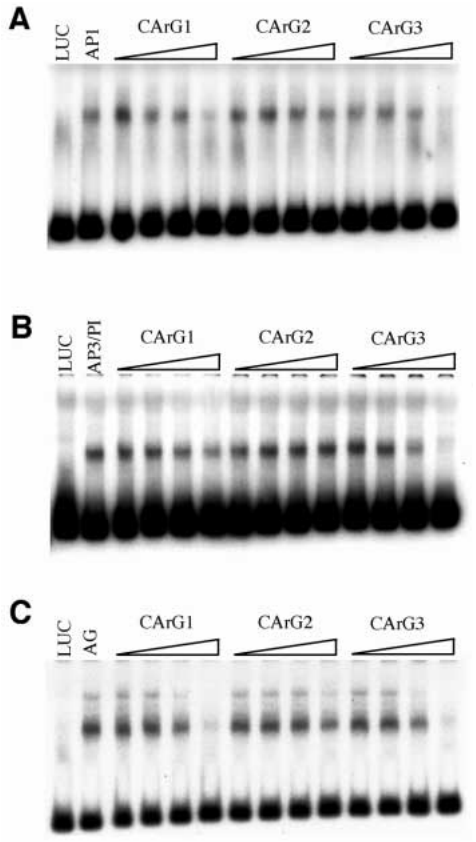

Fig. 6. Competition assays with CArG Boxes 1, 2 and 3. EMSAs showing the effects of using increasing amounts of CArG box sequences on (A) AP1, (B) AP3/PI, and (C) AG binding to the VV fragment. Reticulocyte lysate translation products were incubated with labeled VV fragment in the presence of cold oligo containing CArG boxes 1, 2, or 3. The wedge indicates increasing amounts of 10, 100, 1000 and 10000 fold molar excess of competitor DNA. Control lanes include Luciferase (LUC) translation reaction as a non-specific binding control, and binding in the absence of competitor DNA.

co-translation and subsequent binding assays of the AP3 and PI proteins showed that these proteins were able to bind as a complex to the AB and VV fragments of the *AP3* promoter (Fig. 5). In addition, AP1 and AG proteins could also bind to the AB and VV fragments. The AB fragment corresponds to sequences from −556 to −398 which is within the *cis*-acting region required for maintenance of stamen expression. The VV fragment, from −227 to −83, corresponds to the region required for the initiation and maintenance of *AP3* expression in the petals and stamens (Fig. 5).

The ability of AP1, AP3/PI and AG to bind to individual CArG box motifs within the VV fragment was tested using competition assays. Oligonucleotides corresponding to each of the CArG box sequences were synthesized and used to compete for binding of AP1, AP3/PI and AG to the radiolabelled VV fragment. Increasing concentrations of unlabelled CArG box 1 and CArG box 3, but not CArG box 2, were able to compete for binding in EMSA (Fig. 6). AP1 protein binding to the VV fragment was competed away by similar molar quantities of CArG1 and CArG3, indicating that AP1 has a similar affinity for these binding sites. AG also displays approximately equivalent affinities for binding to CArG1 and CArG3. Binding of the AP3/PI heterodimer, however, was competed away by CarG3 at a lower concentration than CArG1, indicating that AP3/PI has a higher affinity for CArG3 than for CarG1.

DISCUSSION

We have shown that the promoter sequences from −727 to +1 are sufficient to confer all aspects of the normal *AP3* expression pattern on the *uidA* reporter gene product. Based on GUS expression patterns conferred by different fragments of the *AP3* promoter, we have shown that this promoter is modular, in that it contains multiple *cis*-acting elements which control different temporal and/or spatial subsets of the *AP3* expression pattern. The locations of these *cis*-acting elements are summarized in Fig. 2. In addition, we have begun to define the *trans*-acting factors which may play a role in mediating transcriptional expression from these different elements.

Initiation of *AP3* expression

The initial phase of *AP3* expression, from stages 3 to 5, appears to depend on at least two discrete *cis*-acting elements. These 'early' elements map to the region from −224 to −83 (proximal early element), and between −727 and −615 (distal early element). Tilly et al. (1998) report that there is no difference in GUS expression between their −650 and −496 constructs, suggesting that the −650 fragment does not contain the entire distal early element. The proximal early element is capable of conferring a low level of early expression which is dramatically enhanced by the addition of the distal early element. Expression from the distal early element is contingent upon the presence of the proximal early element, since deletions which remove the proximal early element prevent early expression driven by the distal early element. While the distal early element is beyond the region of similarity between the *BobAP3* and *AP3* promoters, it contains several short stretches of homology with the *BobAP3* promoter which may define the binding sites of *trans*-acting factors required for early expression (Fig. 1A).

Several genes appear to be required for the initiation of *AP3* expression, including *AP1*, *UFO* and *LFY* (Weigel and Meyerowitz, 1993; Levin and Meyerowitz, 1995; Lee et al., 1997). Because we do not observe any expression from the 5D6 construct in a *ufo* mutant background, UFO must be required for *AP3* transcriptional activation from sequences contained within the region from −328 to +1. UFO may be involved in *AP3* transcription directly, or may regulate other factors required for *AP3* transcription. Since the effects of *UFO* on *AP3* expression appear to be dependent on *LFY* function (Lee et al., 1997), *LFY* could encode a cofactor for UFO in *AP3* transcriptional activation.

Site-directed mutageneses of CArG boxes 1 and 3 in a synthetic *AP3* promoter context have suggested that CArG box 1 corresponds to a binding site for one or more activators, while CArG box 3 appears to be a binding site for a repressor that acts early, from stages 3 to 5 (Tilly et al., 1998). The *AP1* gene encodes a MADS-domain containing transcription factor, and we have shown that the AP1 gene product can bind to CArG

boxes 1 and 3. Binding of AP1 to CArG box 3 has also been demonstrated by Riechmann and coworkers (Riechmann et al., 1996). Although in vitro binding data may not accurately reflect in vivo function, it is possible that AP1 may regulate early *AP3* expression in part by binding to these two CArG box motifs. Although the loss of *AP1* function alone does not disrupt early *AP3* expression (Fig. 4C and Weigel and Meyerowitz, 1993), *AP1* is clearly required for activation of *AP3*, since plants doubly mutant for *lfy* and *ap1* display no detectable *AP3* expression (Weigel and Meyerowitz, 1993). By stage 3, *AP1* expression becomes restricted from the center of the meristem, and during this period AP1 in combination with other cofactors may activate early *AP3* expression, possibly by binding to CArG box 1. In addition, since *AP1* is expressed throughout the meristem until stage 3, AP1 may act in combination with other cofactors to repress inappropriate *AP3* expression in the first and fourth whorls, perhaps by direct binding to CArG box 3. However, since we do not see ectopic expression of the 5D6 construct in an *ap1-1* mutant background, other factors presumably must be acting to repress first and fourth whorl *AP3* expression. One such factor could be the product of the *CAULIFLOWER* gene, which has been shown to be a redundant paralog of *AP1* (Bowman et al., 1993; Kempin et al., 1995).

Using our promoter-GUS fusions, we have also demonstrated that the initial phase of *AP3* expression is actually asymmetric. We can first detect expression in a 'C' shape at early stage 3, centered at the abaxial side of the developing floral bud and gradually forming a ring of expression in the presumptive petal and stamen primordia by late-stage 3 (Fig. 3B,C). Asymmetry in the *Arabidopsis* flower bud is apparent in the development of sepal primordia, in that the abaxial sepal primordium arises first, but petal and stamen primordia arise symmetrically (Hill and Lord, 1989). This asymmetric *AP3* expression pattern implies that there is an underlying asymmetry in petal and stamen initiation, and may reflect the action of genes whose homologs have been shown to play a role in dorsal-ventral asymmetry in flowers from other species (Luo et al., 1996; Almeida et al., 1997).

AP3 petal-specific expression

By stage 6, *AP3* expression can be seen in the developing petal and stamen primordia (Jack et al., 1992). We have mapped the *cis*-acting elements required for petal-specific expression of *AP3* to within the region from −224 to −83. Furthermore, fusion of the fragment from −328 to +1 to the diphtheria toxin A-chain gene is sufficient to confer petal-specific expression and consequent ablation of these organs in transgenic *Arabidopsis*.

At least three genes, *AP1*, *AP3* and *PI*, appear to be required for high levels of second whorl expression at stage 6 from the 5D6 transgene. Plants mutant for *ap1-1* have greatly reduced expression from the 5D6 transgene at stage 6. It is possible that the requirement for *AP1* function at this stage is mediated directly through binding to CArG box 1 and/or CArG box 3. Alternatively, *AP1* may be acting more indirectly, since the effects of the *ap1-1* mutation on 5D6 expression correlate with the lack of second whorl organs that is observed at this stage in *ap1-1* flowers (Irish and Sussex, 1990; Bowman et al., 1993). Mutations in either *AP3* or *PI* also abolish expression from the 5D6 transgene at stage 6. These auto- and cross-regulatory *AP3* and *PI* functions could be reflecting the binding of a heterodimeric AP3/PI protein complex to the CArG1 and/or CArG3 sequences contained within this region of the promoter.

The loss of *AG* function leads to weak and relatively late ectopic GUS expression in the third whorl of *ag-1* mutant flowers containing the 5D6 transgene. This observation suggests that *AG* may act as a negative regulator of *AP3* expression in the third whorl. The difference in 5D6 expression between the second and third whorls in *ag-1* mutant flowers suggests that despite their apparent morphological similarities, second and third whorl petals develop by qualitatively different mechanisms. These differences are not only manifested at the gene expression level, but are also apparent at the ontogenetic level. Third whorl organs in *ag-1* mutant flowers initially develop with a cell division pattern similar to that of stamens, and only subsequent to stage 7 do these organs develop in a manner more similar to that of petals (Crone and Lord, 1994).

AP3 stamen-specific expression

Two regions, from −556 to −374 (distal stamen element) and from −328 to −83 (proximal stamen element), are both required to confer the full spectrum of stamen-specific *AP3* expression. These elements are both required at stage 6 for all aspects of stamen expression, while later in development, expression in filaments, connectives and throughout the anther appear to be regulated independently by these elements. This suggests that there are tissue-specific factors which mediate late aspects of *AP3* expression in the various stamen tissues.

It is intriguing that the only MADS-domain binding sites which we have defined correspond to the regions which we have shown to be critical for *AP3* expression by deletion analysis. We have found that the AP1, AP3/PI and AG proteins are all capable of binding to sequences in the proximal stamen element and in the AB fragment which contains most of the distal stamen element. Consensus binding sites have not yet been defined for AP1 or AP3/PI, and analyses of the AB fragment fail to yield sequences similar to the consensus binding sites defined for AG (Huang et al., 1993; Shiraishi et al., 1993). However, the AB fragment does contain numerous A/T rich regions which may be involved in MADS-domain protein binding. These observations suggest that *AP3* stamen-specific expression may depend in part on the binding of these, or other MADS-domain containing proteins, to specific sequences within the distal and proximal stamen elements.

Other *trans*-acting factors regulating *AP3* expression

We did not detect any ectopic GUS expression patterns in our promoter dissection analysis. However, mutations in several loci are known to result in ectopic *AP3* expression. Mutations in *SUP* result in ectopic fourth whorl *AP3* expression, while mutations in *CURLY LEAF (CLF)* result in *AP3* expression in the leaf (Bowman et al., 1992; Sakai et al., 1995; Goodrich et al., 1997). *CLF* encodes a product with similarity to the Polycomb-group of proteins which are thought to be required to maintain particular transcriptional states by altering chromatin structure (Goodrich et al., 1997). We can suggest three possibilities for why we did not detect any ectopic expression patterns that might correlate with the loss of *SUP*

or *CLF* function. First, there could be multiple redundant binding sites for SUP or CLF proteins, or for proteins whose expression is regulated by *SUP* or *CLF*. Elimination of one binding site in our promoter deletion analyses would not be sufficient to alleviate the negative regulatory effects of binding to the remaining sites. Second, it is possible that binding sites for these proteins reside within the sequences from −83 to +37. Finally, it is possible that the SUP and CLF gene products function at the posttranscriptional level to regulate *AP3* mRNA stability.

The strength of MADS-domain containing transcription factor binding may be influenced in vivo by the binding of cofactors to adjacent sites, or cooperative interactions between transcription factors. Substitution of the amino-terminal half of the AP1, AP3, PI and AG MADS domains with heterologous MADS domains has little effect on binding specificities or on the phenotypes produced by ectopic expression (Riechmann and Meyerowitz, 1997). In addition, analyses of MADS domain containing proteins in yeast and mammals have shown that their binding specificity can be significantly modified by binding to bHLH or homeodomain-containing cofactors (Molkentin et al., 1995; Black et al., 1996; Mead et al., 1996). Another possible class of cofactors are the MADS-box containing *AGL* genes, several of which are expressed during flower development (Ma et al., 1991; Huang et al., 1995; Savidge et al., 1995). These observations have suggested that selective interactions with cofactors and the specificity of cofactor-DNA binding may influence the in vivo function of these floral homeotic proteins. The fact that CArG boxes 1 and 3 are not completely conserved between Arabidopsis and Brassica, yet lie embedded in regions of sequence which show a high degree of similarity, may reflect the conservation of cofactor binding sites which modulate MADS-domain protein binding.

These results have demonstrated that specific temporal and spatial *cis*-acting elements exists within the *AP3* promoter, and have uncovered some previously undescribed characteristics of *AP3* expression. Furthermore, while the binding studies suggest a role for several MADS-domain containing proteins in regulating AP3 expression, it is likely that their in vivo role is modified by interactions with cofactors. The identification of such factors may help to shed light on the underlying molecular mechanisms by which cells in the floral meristem become regionalized.

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