Developmental Biology **216,** 260–264 (1999)

Redundant Enhancers Mediate Transcriptional Repression of *AGAMOUS* by *APETALA2*

Kirsten Bomblies, Nicole Dagenais, and Detlef Weigel[1]

Plant Biology Laboratory, The Salk Institute for Biological Studies, 10010 N. Torrey Pines Road, La Jolla, California 92037

The floral homeotic gene *AGAMOUS* specifies stamen and carpel fate in the central whorls of *Arabidopsis* flowers. Transcription of *AGAMOUS* RNA is restricted to the center of developing flowers by several, partially redundant negative regulators, one of which is the homeotic gene *APETALA2*. We have identified regulatory elements that mediate transcriptional repression of *AGAMOUS* by *APETALA2* and found that several redundant elements respond independently to loss of *APETALA2* activity. Thus, redundancy at the level of *cis*-regulatory sequences is independent of redundancy at the level of *trans*-regulators. We have also found that only the early, but not the late, effects of *APETALA2* on *AGAMOUS* require the meristem-identity protein LEAFY, a positive regulator of *AGAMOUS*.

***Key Words: Arabidopsis;* flower development; homeotic genes; *AGAMOUS; APETALA2; LEAFY*.**

INTRODUCTION

Flowers of dicotyledonous plants contain four major organ types, sepals, petals, stamens, and carpels, arranged in concentric rings or whorls. The fate of floral organ types is specified by three classes of homeotic genes, A, B, and C, each of which controls organ fate in two adjacent whorls. A function in the first, outer whorl specifies sepal fate; A plus B function in the second whorl, petal fate; B plus C function in the third whorl, stamen fate; and C in the fourth, central whorl carpel fate (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994).

The activity of most homeotic genes is regulated primarily at the transcriptional level, and their RNAs accumulate preferentially in those anlagen and primordia where their genetic activity is required. An example is the *Arabidopsis* C function gene *AGAMOUS (AG),* whose expression in the center of the flower is controlled by several redundantly acting enhancers (Busch *et al.*, 1999). Mutant screens have identified numerous *trans*-acting genes that control *AG* expression, although it is mostly unknown whether the proteins encoded by these genes regulate *AG* directly or indirectly. An exception is the transcription factor encoded by the floral meristem-identity gene *LEAFY (LFY),* which specifies overall floral as opposed to leaf and shoot fate and which is an important positive regulator of *AG* (Schultz and Haughn, 1991; Huala and Sussex, 1992; Weigel *et al.*, 1992; Weigel and Meyerowitz, 1993). LFY binds to an *AG* transcriptional enhancer *in vitro,* and its binding is required for activity of this enhancer *in vivo* (Busch *et al.*, 1999).

Several negative regulators of *AG* have been identified by mutation as well, and some of these act at least partially redundantly (Drews *et al.*, 1991; Bowman *et al.*, 1993; Liu and Meyerowitz, 1995; Goodrich *et al.*, 1997; Chen *et al.*, 1999). The most severe effects are seen in plants mutant for *APETALA2 (AP2),* which encodes a founding member of a new class of DNA-binding transcription factors (Ohme-Takagi and Shinshi, 1995; Okamuro *et al.*, 1997; Riechmann and Meyerowitz, 1998). In *ap2* mutants, *AG* expression begins earlier, the levels of expression are elevated, and the expression domain is expanded toward the periphery (Drews *et al.*, 1991). Despite having severe defects on their own, the phenotype of strong *ap2* mutants becomes even more severe when combined with mutations in *LEUNIG (LUG),* which on its own has similar, but weaker effects as *AP2* (Liu and Meyerowitz, 1995). Since there are both redundant *cis*-acting elements of *AG* and redundant *trans*-acting regulators, we have investigated the possibility that the different *AG* enhancers are regulated by different negative factors.

MATERIALS AND METHODS

All *AG* reporter constructs have been described (Busch *et al.*, 1999). *In situ* hybridization and β-glucuronidase (GUS) staining were performed as described (Parcy *et al.*, 1998; Busch *et al.*, 1999).

[1] To whom correspondence should be addressed. Fax: (858) 558-6379. E-mail: weigel@salk.edu.

0012-1606/99 $30.00

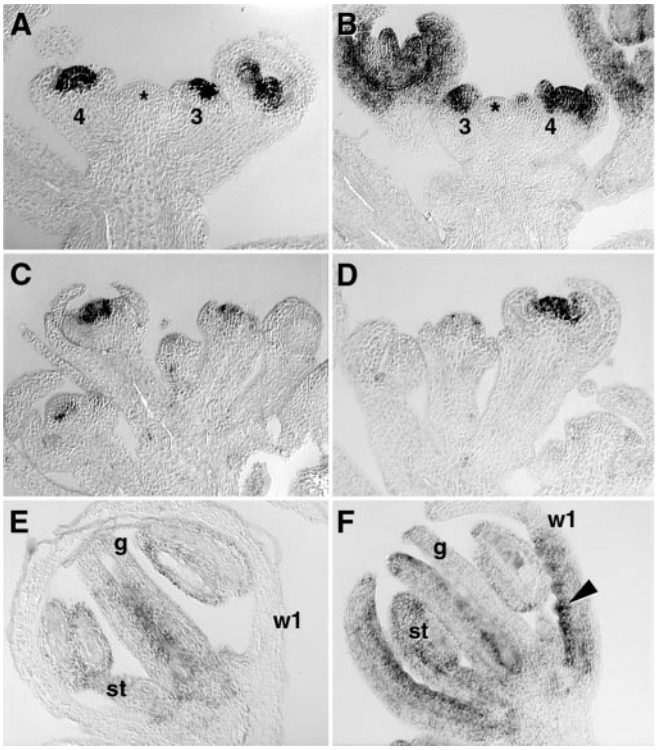


FIG. 1. *AG* RNA localization by *in situ* hybridization. (A) Wild-type apex with young flowers. Numbers indicate floral stages (Smyth *et al.*, 1990). Asterisk indicates shoot apical meristem. (B) *ap2-6* apex with young flowers. Note that *AG* RNA expands into the first-whorl organs. (C) *lfy-12* flowers. (D) *lfy-12 ap2-6* flowers. (E) Stage 9 wild-type flower. (F) Stage 9 *ap2-6* flower. Note expression on the adaxial side of first-whorl organ, which is carpelloid and is forming ovules (arrowhead). w1, first-whorl organ; st, stamen; g, gynoecium. All panels are at the same magnification.

For whole-mount analysis, GUS-stained material was cleared in 70% ethanol, dissected, and viewed in 20% ethanol, 20% glycerol under differential interference contrast.

RESULTS AND DISCUSSION

Because *ap2* mutations have the most severe effects among the mutations described to cause derepression of *AG* in flowers, we focused in this study on *AP2*. Our *AG* reporter lines, in which *AG* genomic sequences are linked to the −46-bp cauliflower mosaic virus 35S promoter driving the GUS reporter, were generated in the Columbia background (Busch *et al.*, 1999). We used therefore the strong *ap2-6* allele, which was also induced in Columbia (Kunst *et al.*, 1989). Similarly to what has been described for *AG* expression in the strong *ap2-2* allele (Drews *et al.*, 1991), which is in the Landsberg *erecta* background, expression of *AG* RNA was detected earlier and in a broader domain in *ap2-6* flowers (Figs. 1A and 1B). In addition, *AG* RNA levels were increased in the placenta of carpels at later stages (Figs. 1E and 1F).

Transcriptional regulation of *AG* requires enhancer sequences that span most of the second intron (Sieburth and Meyerowitz, 1997). This intron, which largely coincides with a 3-kb *Hin*d III restriction fragment, contains several, partially redundant enhancers. Specifically, two nonoverlapping fragments can independently drive early expression of GUS in the center of the flower (Busch *et al.*, 1999) (Fig. 2), while expression at later stages of flower development requires enhancer sequences at the 5′ end of this intron. To determine whether all or only a subset of enhancers are regulated by *AP2*, we crossed several *AG* reporter constructs into *ap2-6* plants (Fig. 2).

Regulation of Early Acting AG Enhancers by AP2

The KB9 reporter, which carries the complete 3-kb *Hin*d III *AG* fragment, shows a GUS expression pattern that mimics both the early and the late expression of *AG* RNA (Busch *et al.*, 1999). GUS expression of the KB9 reporter in *ap2-6* mutants was very similar to that of endogenous *AG* RNA in *ap2-6* mutants (Figs. 3A and 3B). This finding confirms that *AP2* acts through sequences located in the second intron of *AG*, as previously demonstrated with *AG* promoter fusions that either contained or lacked the second intron (Sieburth and Meyerowitz, 1997). Three shorter constructs, KB14, KB18, and KB31, which also show strong early expression in wild type, are similarly expanded in *ap2-6* (Figs. 3C–3H). Importantly, *AG* sequences in KB14 and KB31 do not overlap, indicating that the 5′ and 3′ early enhancers are independently regulated by *AP2*.

KB30 contains part of the 3′ enhancer and its activity in wild type is much weaker than that of KB31, although the early pattern of KB30 appears to be largely unchanged when compared to KB31 (Busch *et al.*, 1999). Activity of KB30 was increased in *ap2-6* (Figs. 4A and 4B), indicating that *AP2* can act independently of regulators that control the overall expression level of *AG*. A similar effect was seen with the overlapping construct KB33, whose activity is even weaker than that of KB30 (Fig. 2). The effect of the *ap2-6* mutation on KB30 and KB33 was similar to that of the activated version of *LFY*, *LFY:VP16*, which is also able to increase expression of *AG* reporter constructs that are on their own only weakly expressed (Busch *et al.*, 1999). However, in contrast to *LFY:VP16*, *ap2-6* could not restore expression of the smaller constructs KB24 and KB28, which retain LFY-binding sites, but are not active on their own in wild type (Fig. 2) (Busch *et al.*, 1999).

Requirement of LFY Activity for Early Regulation of AG by AP2

An important positive regulator of *AG* is the transcription factor encoded by the floral meristem-identity gene *LFY* (Weigel and Meyerowitz, 1993; Busch *et al.*, 1999). *AP2* can act independently of *LFY*, since the *lfy ap2* double mutant phenotype is different from that of *lfy* single mutants (Huala and Sussex, 1992; Weigel *et al.*, 1992). However, *AG* expression is not obviously increased in *lfy-12*

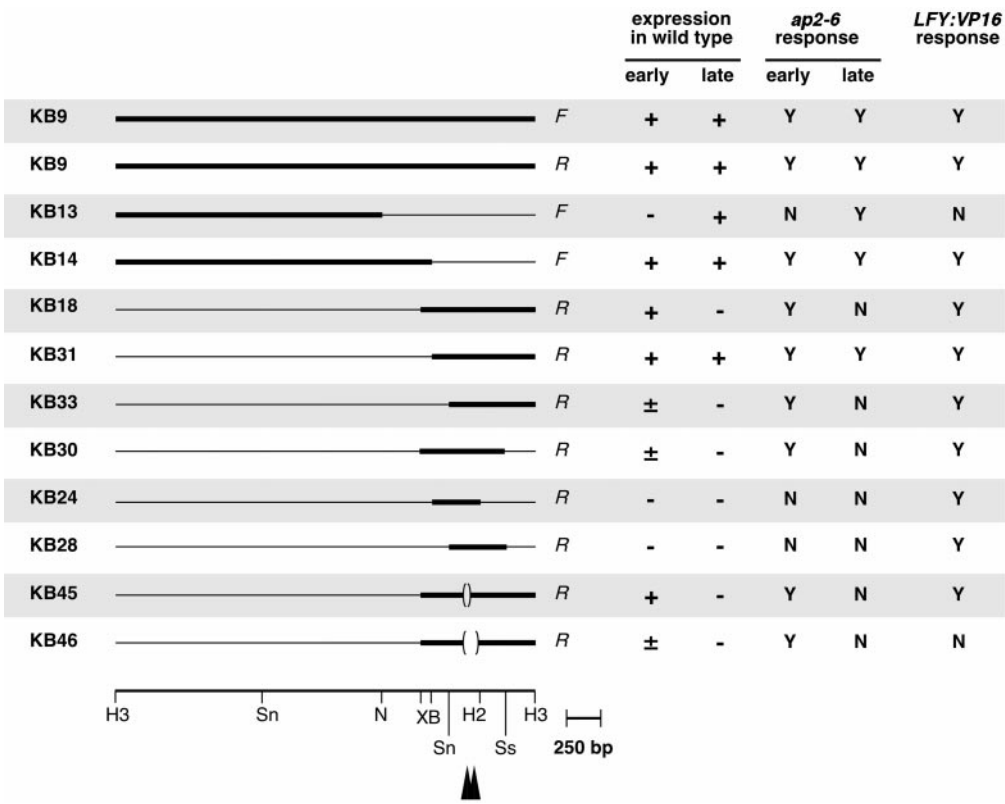


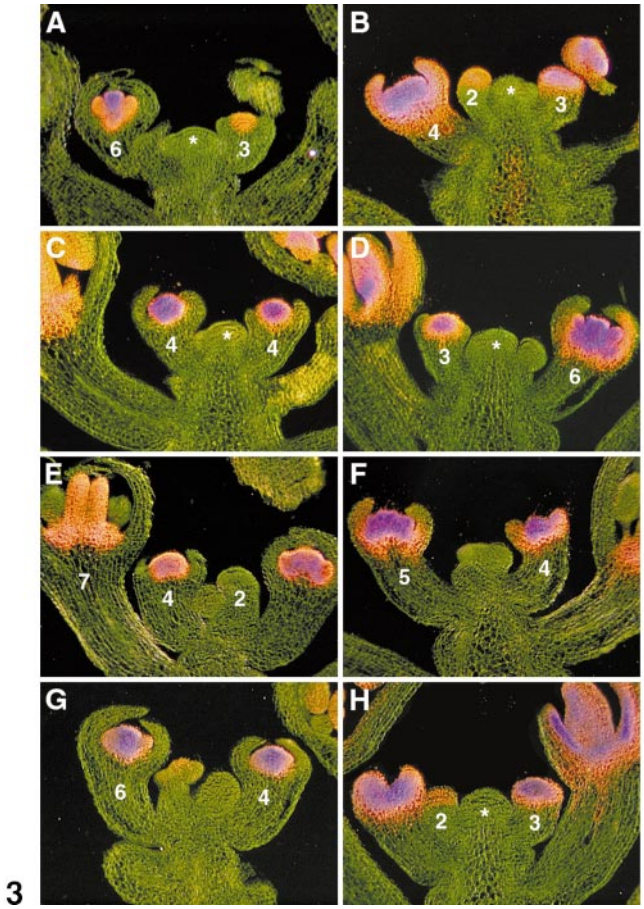
FIG. 2. Diagram of reporter constructs carrying *AG* genomic fragments in front of a minimal 35S promoter driving GUS. KB9 contains the 3-kb *Hin*dIII fragment that is largely identical with the second *AG* intron (Yanofsky *et al.*, 1990; Busch *et al.*, 1999). F (forward) and R (reverse) indicate that the 3′ or 5′ end, respectively, was closest to the minimal 35S promoter. Early, expression during stages 3 to 5; late, expression after stage 8. +, ±, or −, whether and how well a line stained in an *AG*-typical pattern. The *ap2-6* response is indicated by Y (yes) or N (no). For comparison, the *LFY:VP16* response is indicated (Busch *et al.*, 1999). Two LFY-binding sites are indicated by arrowheads below the restriction map. The deletion in KB45 removes one of these sites; the deletion in KB46 removes both sites (Busch *et al.*, 1999). Restriction sites: B, *Bam*HI; H2, *Hin*cII; H3, *Hin*dIII; N, *Nla*IV; Sn, *Sna*BI; Ss, *Ssp*I; X, *Xba*I.

ap2-6 double mutants when compared to *lfy-12* single mutants (Figs. 1C and 1D), indicating that the effects of *AP2* on *AG* are at least partially dependent on *LFY* activity. Similar results to those observed for endogenous *AG* RNA were seen with the *AG* reporters KB14 and KB18, whose activities were very much reduced in both *lfy-12* and *lfy-12 ap2-6* mutants (not shown).

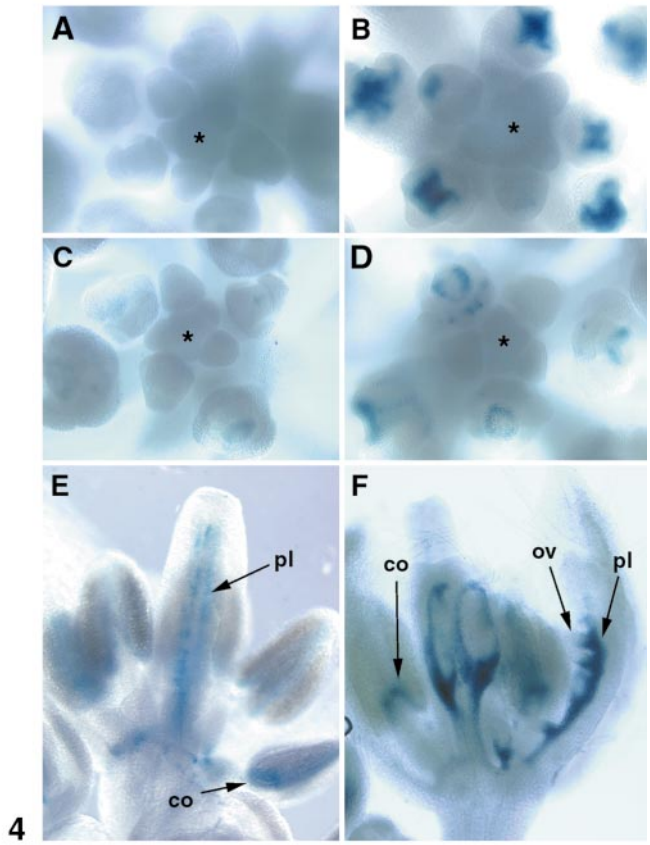
To investigate whether the effects of *AP2* on *AG* depend on interaction of LFY with *AG* sequences, we crossed *ap2-6* to the KB46 reporter, which contains the same *AG* genomic fragment as KB18, but carries an internal 77-bp deletion that removes two LFY-binding sites (Busch *et al.*, 1999). Activity of KB46 in wild type is very much reduced when compared to KB18, and its activity cannot be restored by *LFY:VP16* (Busch *et al.*, 1999). In contrast to *LFY:VP16*, we observed a slight response of KB46 to *ap2-6* (Figs. 4C and 4D), although the response was attenuated when compared to KB30, which is a short construct that contains the LFY-binding sites but is otherwise only very weakly active in wild type (see above). This result suggests both LFY-dependent and LFY-independent effects of *AP2* on *AG*.

Regulation of a Late Acting AG Enhancer by AP2

AG is expressed throughout much of flower development (Yanofsky *et al.*, 1990; Bowman *et al.*, 1991; Drews *et al.*, 1991). Among the reporters that had an *AG*-typical pattern, we found several that were expressed only during early stages of flower development. In contrast, we did not find reporters that showed exclusively strong *AG*-typical expression during later stages of flower development (Fig. 2). However, a candidate for a reporter containing some late acting regulatory elements is KB13. Most lines of this reporter, which lacks the 3′ most 357 bp present in KB14, showed no early GUS activity. Expression in later stages was weak and restricted to specific tissues within the developing stamens and carpels, particularly the connective and anther walls of the stamens, and the placenta of the carpel (Fig. 4E), which overlap with the sites of endogenous *AG* RNA expression (Fig. 1F) (Bowman *et al.*, 1991). Stronger KB13 lines showed in addition ectopic GUS expression in the stem, in the shoot apical meristem, and in early flowers (Busch *et al.*, 1999).



3



4

FIG. 3. Examples of GUS expression in *AG* reporter lines. Wild type is on the left and *ap2-6* is on the right. (A, B) KB9; (C, D) KB14; (E, F) KB18; (G, H) KB31. Apices were stained for GUS activity using X-gluc, embedded, sectioned, and viewed under dark-field illumination. Weak staining appears orange, and strong staining appears pink to purple. All panels are at the same magnification.

FIG. 4. Examples of GUS expression in *AG* reporter lines. Wild type is on the left and *ap2-6* is on the right. (A–D) Apices with young flowers. Panels are at the same magnification. The shoot apical meristems are indicated by asterisks. (A, B) KB30; (C, D) KB46. (E, F) Partially dissected older flowers (stage 9 to 10) of plants carrying the construct KB13. Apices and flowers were stained for GUS activity using X-gluc, dissected, and viewed under differential interference contrast. Both panels are at the same magnification. co, connective tissue; pl, placenta; ov, ovules.

To investigate the effects of *AP2* on the late acting sequences in KB13, we chose a KB13 line that was only weakly expressed in wild-type flowers, without any obvious ectopic expression during early stages (Fig. 4E). As in wild type, no early GUS expression was observed when this line was introduced into the *ap2-6* background (not shown). In contrast, later expression in stamens and carpels increased. Notably, increased placental expression was observed both in the normal fourth-whorl gynoecium and in the ectopic first-whorl carpels (Fig. 4F). This pattern was similar to what we observed for endogenous *AG* RNA in later flowers of *ap2-6* mutants (Fig. 1F). These results demonstrate that despite the early ectopic expression seen in stronger KB13 lines, KB13 retains a late *AP2* response. These results also demonstrate that there are separable elements mediating the early and late effects of *AP2* on *AG*.

Since KB13 expression was not increased in *LFY:VP16* (Busch *et al.*, 1999), which has a floral phenotype similar to that of *ap2* mutants (Parcy *et al.*, 1998), we conclude that the effect of *AP2* on KB13 does not simply reflect changed tissue identity, but rather that the effect of *AP2* on KB13 is specific and relatively direct. The lack of a *LFY:VP16* response in KB13 also indicates that *AP2* can affect late expression of *AG* independently of *LFY*.

CONCLUSIONS

The homeotic gene *AG* is under the control of several negative regulators that restrict *AG* expression to the center of the flower, from which the stamens and carpels arise (Drews *et al.*, 1991; Bowman *et al.*, 1993; Liu and Meyer-

owitz, 1995; Goodrich *et al.*, 1997; Chen *et al.*, 1999). At least some of these regulators prevent also precocious floral expression of *AG* before stage 3 of development (Drews *et al.*, 1991; Liu and Meyerowitz, 1995). Because there are both redundant *cis*-regulatory sequences as well as redundant *trans*-regulators for *AG*, we have asked whether the *AG* regulator *AP2* regulates only a subset of *AG* enhancers, with a different set of *AG* enhancers being regulated by other regulators such as *LUG*. We have shown that this is not the case and that *AP2* is equally required for negative regulation of several, partially redundant enhancers that control *AG* expression at different stages of flower development. We have also shown that early and late effects of *AP2* on *AG* expression can be separated. Finally, we have demonstrated that early *AP2* action on *AG* requires the positive regulator *LFY*. It remains to be determined whether AP2 binds directly to *AG* enhancer sequences, as LFY does (Busch *et al.*, 1999), and if so, whether AP2 and LFY interact with each other.

ACKNOWLEDGMENTS

We are grateful to Jasmine T. Nguyen for excellent technical assistance. We thank Max Busch and Ray Hong for discussion and the NSF-supported Arabidopsis Biological Resource Center at Ohio State University for seeds. This study was supported by a grant from the U.S. Department of Energy to D.W., who is an NSF Young Investigator.

REFERENCES

Bowman, J. L., Alvarez, J., Weigel, D., Meyerowitz, E. M., and Smyth, D. R. (1993). Control of flower development in *Arabidopsis thaliana* by *APETALA1* and interacting genes. *Development* **119,** 721–743.

Bowman, J. L., Drews, G. N., and Meyerowitz, E. M. (1991). Expression of the *Arabidopsis* floral homeotic gene *AGAMOUS* is restricted to specific cell types late in flower development. *Plant Cell* **3,** 749–758.

Busch, M. A., Bomblies, K., and Weigel, D. (1999). Activation of a floral homeotic gene in *Arabidopsis*. *Science* **285,** 585–587.

Chen, Q., Atkinson, A., Otsuga, D., Christensen, T., Reynolds, L., and Drews, G. N. (1999). The *Arabidopsis FILAMENTOUS FLOWER* gene is required for flower formation. *Development* **126,** 2715–2726.

Coen, E. S., and Meyerowitz, E. M. (1991). The war of the whorls: Genetic interactions controlling flower development. *Nature* **353,** 31–37.

Drews, G. N., Bowman, J. L., and Meyerowitz, E. M. (1991). Negative regulation of the *Arabidopsis* homeotic gene *AGAMOUS* by the *APETALA2* product. *Cell* **65,** 991–1002.

Goodrich, J., Puangsomlee, P., Martin, M., Long, D., Meyerowitz, E. M., and Coupland, G. (1997). A Polycomb-group gene regulates homeotic gene expression in *Arabidopsis*. *Nature* **386,** 44–48.

Huala, E., and Sussex, I. M. (1992). *LEAFY* interacts with floral homeotic genes to regulate *Arabidopsis* floral development. *Plant Cell* **4,** 901–913.

Kunst, L., Lenz, J. E., Martinez-Zapater, J., and Haughn, G. W. (1989). *AP2* gene determines the identity of perianth organs in flowers of *Arabidopsis thaliana*. *Plant Cell* **1,** 1195–1208.

Liu, Z., and Meyerowitz, E. M. (1995). *LEUNIG* regulates *AGAMOUS* expression in *Arabidopsis* flowers. *Development* **121,** 975–991.

Ohme-Takagi, M., and Shinshi, H. (1995). Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. *Plant Cell* **7,** 173–182.

Okamuro, J. K., Caster, B., Villarroel, R., Van Montagu, M., and Jofuku, K. D. (1997). The AP2 domain of APETALA2 defines a large new family of DNA binding proteins in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **94,** 7076–7081.

Parcy, F., Nilsson, O., Busch, M. A., Lee, I., and Weigel, D. (1998). A genetic framework for floral patterning. *Nature* **395,** 561–566.

Riechmann, J. L., and Meyerowitz, E. M. (1998). The AP2/EREBP family of plant transcription factors. *Biol. Chem*. **379,** 633–646.

Schultz, E. A., and Haughn, G. W. (1991). *LEAFY*, a homeotic gene that regulates inflorescence development in *Arabidopsis*. *Plant Cell* **3,** 771–781.

Sieburth, L. E., and Meyerowitz, E. M. (1997). Molecular dissection of the *AGAMOUS* control region shows that *cis* elements for spatial regulation are located intragenically. *Plant Cell* **9,** 355–365.

Smyth, D. R., Bowman, J. L., and Meyerowitz, E. M. (1990). Early flower development in *Arabidopsis*. *Plant Cell* **2,** 755–767.

Weigel, D., Alvarez, J., Smyth, D. R., Yanofsky, M. F., and Meyerowitz, E. M. (1992). *LEAFY* controls floral meristem identity in *Arabidopsis*. *Cell* **69,** 843–859.

Weigel, D., and Meyerowitz, E. M. (1993). Activation of floral homeotic genes in *Arabidopsis*. *Science* **261,** 1723–1726.

Weigel, D., and Meyerowitz, E. M. (1994). The ABCs of floral homeotic genes. *Cell* **78,** 203–209.

Yanofsky, M. F., Ma, H., Bowman, J. L., Drews, G. N., Feldmann, K. A., and Meyerowitz, E. M. (1990). The protein encoded by the *Arabidopsis* homeotic gene *agamous* resembles transcription factors. *Nature* **346,** 35–39.

Received for publication July 12, 1999
Revised September 9, 1999
Accepted September 14, 1999