Cell, Vol. 92, 93–103, January 9, 1998, Copyright ©1998 by Cell Press

A Homolog of *NO APICAL MERISTEM* Is an Immediate Target of the Floral Homeotic Genes *APETALA3/PISTILLATA*

Robert W. M. Sablowski and Elliot M. Meyerowitz*
California Institute of Technology
Division of Biology 156-29
Pasadena, California 91125

Summary

To understand how homeotic genes affect morphogenesis and differentiation, their target genes must be identified. In *Arabidopsis* flowers, the homeotic protein heterodimer APETALA3/PISTILLATA is necessary for petal and stamen formation. Here, AP3/PI function was put under posttranslational control to analyze its immediate effect on the floral mRNA population, with indirect effects blocked by cycloheximide. Using differential display, a target gene of AP3/PI was identified (*NAP: NAC-LIKE, ACTIVATED BY AP3/PI*), which is homologous to genes required for meristem establishment and separation of floral organs. The expression pattern of *NAP* and the phenotypes caused by its misexpression suggest that it functions in the transition between growth by cell division and cell expansion in stamens and petals.

Introduction

Homeotic mutations have provided evidence for modularity in the development of both animals and plants; the identity of discrete body parts can be transformed by loss of function or ectopic expression of homeotic genes (reviewed by Krumlauf, 1994; Lawrence and Morata, 1994; Weigel and Meyerowitz, 1994). Because these encode transcription factors (homeodomain proteins in animals and mostly MADS-domain proteins in plants), their function at the molecular level must be the regulation of a set of genes required for the formation of a developmental module. Few of these target genes are known in any organism, mainly because their systematic identification has proven technically difficult (reviewed by Graba et al., 1997).

Identifying genes subordinate to homeotic genes is important not only in understanding how homeotic genes work but also in addressing general questions on the nature and evolution of modules of gene expression used in development. Plants, for example, contain large families of MADS-box genes, which probably diversified from a small family present in the common ancestor of vascular plants (Münster et al., 1997). How much of their functional diversification involved recruitment of new target genes, and to what extent have target genes coevolved with their regulators? Do floral homeotic genes directly control structural genes required for floral organ formation, or is that function delegated to intermediary regulators? Do intermediary regulators control batteries of target genes also corresponding to functional modules, which can be recalled in different contexts during development? These questions cannot be answered before knowledge about the identities and functions of target genes is accumulated.

Floral homeotic genes have been best characterized in *Arabidopsis*, snapdragon, and petunia (Yanofsky, 1995; reviewed in Riechmann and Meyerowitz, 1997b). In all three species, flowers are composed of four concentric rings (whorls) of organs, with sepals in the first, outermost whorl, followed by petals, stamens, and carpels in whorls 2, 3, and 4, respectively. The well-established ABC model explains how floral homeotic genes act combinatorially to specify each of the four organ identities (Bowman et al., 1991; Coen and Meyerowitz, 1991). Class A genes function in the first two whorls; class B, in whorls 2 and 3; class C, in whorls 3 and 4. Class A and C functions are mutually exclusive; loss of either causes the other to extend to the whole flower. Sepals are formed in whorls expressing class A genes; petals require A and B, stamens are formed by B combined with C, and C activity alone is required to form carpels.

In *Arabidopsis*, there are two known class B genes: *APETALA3 (AP3)* and *PISTILLATA (PI)*. Loss of function of either causes the same phenotype; petals are replaced by sepaloid organs and stamens are replaced by carpelloid organs, which often fuse with the fourth whorl carpels (Bowman et al., 1989; Hill and Lord, 1989; Jack et al., 1992). Both *AP3* and *PI* encode proteins belonging to the MADS box family of transcription factors and are expressed throughout the development of petals and stamens (Jack et al., 1992; Goto and Meyerowitz, 1994). Combined ectopic expression of AP3 and PI is sufficient to provide B activity anywhere within a flower (Jack et al., 1994; Krizek and Meyerowitz, 1996a). The AP3 and PI proteins bind DNA in vitro only as an AP3/PI heterodimer (Riechmann et al., 1996a), which likely acts as a master regulator, necessary and sufficient (within a flower with intact A and C functions) to set in motion the programs of gene expression required for petal and stamen development.

Here, to identify AP3/PI target genes, we looked for mRNAs whose levels were affected by posttranslational activation of AP3/PI function in floral organs, using the strategy outlined below. With this approach, we have started to uncover the genes and functions controlled by AP3/PI during floral morphogenesis.

Results

Strategy for Identifying Genes Regulated by AP3/PI

The approach was based on detection of mRNAs whose levels were affected by activation of AP3/PI function in floral organs. To reduce the complexity of the mRNA populations to be compared, we used flowers producing only sepals (B and C functions removed by the *ap3-3* and *ag-3* mutations; Figure 1, top). Ubiquitously inducible B function was added, using the viral 35S promoter to direct constitutive expression of PI (*35S::PI*) combined with ubiquitous expression of a steroid-regulated version of AP3 (*35S::AP3-GR*). The latter was created by

*To whom correspondence should be addressed.

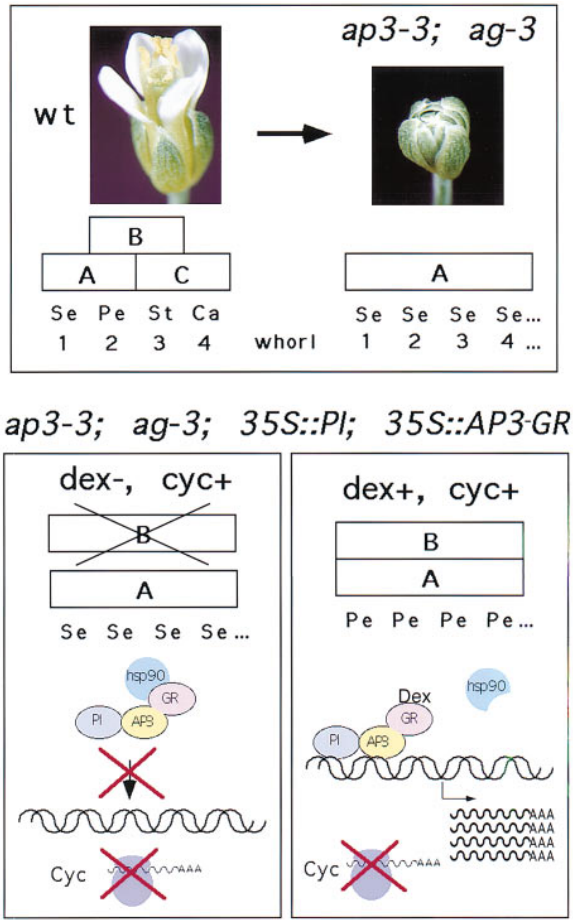

Figure 1. Strategy for Identifying AP3/PI Target Genes

(Top) wild-type and *ap3-3; ag-3* mutant flowers. The scheme below represents the domains of A, B, and C functions and the corresponding organ identities. *ag-3* flowers are indeterminate, continuously producing new whorls of organs. Se: sepals; Pe: petals; St: stamens; Ca: carpels.

(Bottom) rationale of the screen for AP3/PI target genes. In *ap3-3; ag-3; 35S::PI; 35S::AP3-GR* flowers, dexamethasone (dex) should release AP3-GR from hsp90 and activate B function in all floral organs by allowing AP3-GR/PI to activate transcription (right). Protein synthesis was blocked (represented by the crossed ribosome) with cycloheximide (cyc) to avoid indirect gene regulation by the products of AP3/PI target genes. mRNAs accumulated after dexamethasone treatment are identified by comparison with controls treated with cycloheximide alone (left).

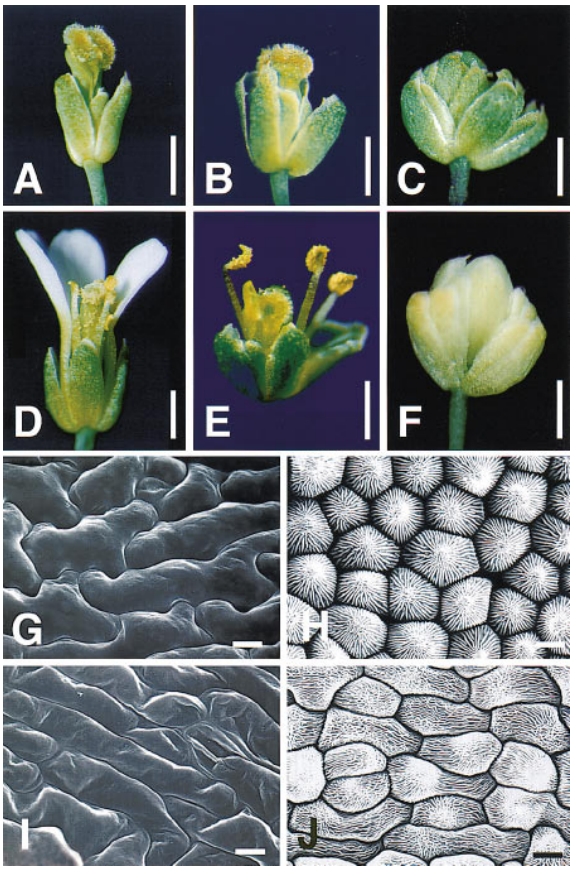

Figure 2. Steroid-Inducible AP3 Function

Individual flowers were soaked once every day for 10 days with 0.015% v/v Silwet L-77 (OSI Specialties) containing 0.1% ethanol alone (mock treatment) or 0.1% ethanol and 5 μM dexamethasone. Four days after the last treatment, flowers were photographed or fixed for scanning electron microscopy according to Bowman et al., 1989. (A–F), bar = 1 mm; (G–J), bar = 10 μm.

(A) *ap3-3* mutant flower, untreated; (B) *ap3-3; 35S::AP3-GR* flower, mock-treated; (C) *ap3-3; ag-3; 35S::AP3-GR; 35S::PI* flower, mock-treated; (D) wild-type L-*er* flower, untreated; (E) *ap3-3; 35S::AP3-GR* flower, dexamethasone-treated; (F) *ap3-3; ag-3; 35S::AP3-GR; 35S::PI* flower, dexamethasone-treated; (G and H) scanning electron microscopy view of the adaxial surface of untreated wild-type sepals (G) or petals (H); (I and J): adaxial surface of *ap3-3; ag-3; 35S::AP3-GR; 35S::PI* floral organs, mock-treated (I) or treated with dexamethasone (J).

fusion of the AP3 protein with the hormone-binding domain of the rat glucocorticoid receptor (GR). This method, previously shown to work in plants (Lloyd et al., 1994; Simon et al., 1996; Aoyama and Chua, 1997), had an important advantage in our case: activation of AP3 function by exogenous steroid should occur by release of hsp90 bound to the fusion protein (Dalman et al., 1991, and references therein), a process that does not require new protein synthesis (Figure 1, bottom). Thus, the immediate effects of AP3/PI activation on the floral mRNA population could be analyzed by differential display (Liang and Pardee, 1992) while indirect effects were blocked by a protein synthesis inhibitor.

AP3-GR Mimics *AP3* Function

Before AP3-GR could be used in the search for *AP3/PI* target genes, it was essential to demonstrate that the fusion protein could restore AP3 function in the null *ap3-3* mutant. In the absence of steroid hormone, *ap3-3; 35S::AP3-GR* flowers were indistinguishable from *ap3-3* mutants (Figures 2A and 2B). After dexamethasone treatment, B function was rescued in the third whorl, varying from formation of free–standing carpelloid organs to fully functional stamens, and fourth whorl carpelloid organs were unfused (Figure 2E). A similar phenotype was caused by ubiquitous expression of wild-type AP3 (*35S::AP3*) in an *ap3-3* background (Jack et al., 1994). Also similar to *35S::AP3* (Jack et al., 1994; Krizek and

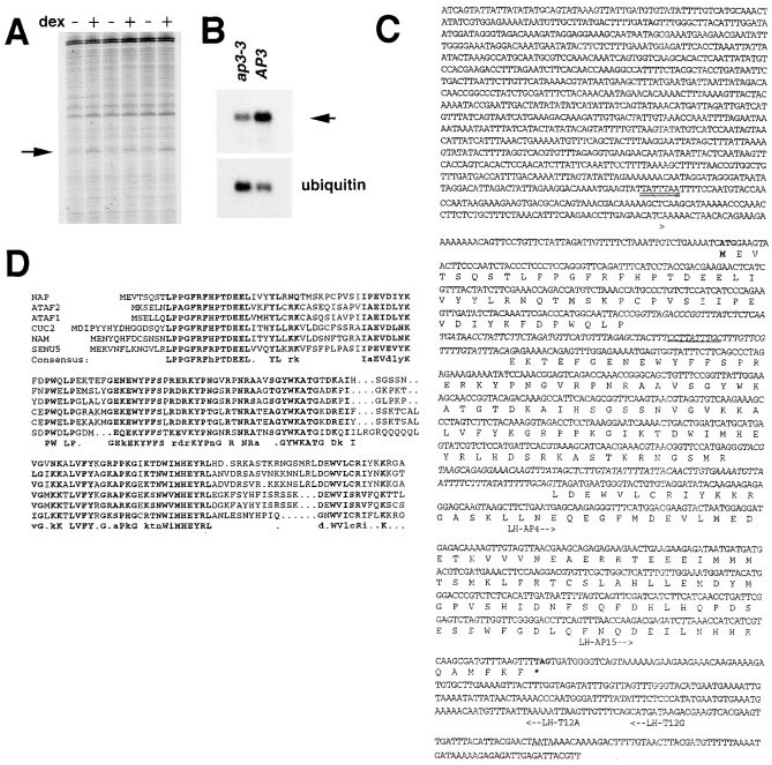

Figure 3. Identification of a mRNA Up-Regulated by AP3-GR

(A) Differential display performed as described in Experimental Procedures, in this case with primers LH-AP4 and LH-T12G. Each lane corresponds to one independent treatment with cycloheximide alone (-) or cycloheximide combined with dexamethasone (+). The arrow points to the band corresponding to the AP3-GR–activated mRNA.

(B) RNA blot [approximately 2 μg poly(A)$^+$ RNA per lane] probed with the differential display product shown in (A) (arrow), comparing mRNA levels in wild-type and *ap3-3* inflorescences. The lane corresponding to wild-type flowers contained slightly less RNA, as shown by the ubiquitin control.

(C) Sequence of the gene corresponding to the differential display product shown in (A). The beginning of the longest cDNA is indicated by an arrowhead. Start and stop codons are boldfaced; intron sequences are italicized. Sites primed during differential display are indicated by arrows and primer name. The sites primed by LH-T12G and LH-T12A may correspond to polyadenylation sites; a consensus polyadenylation site present downstream is also indicated by a dotted line. A CArG–box-like sequence (see Riechmann and Meyerowitz, 1997b) present in the first intron is underlined; a putative TATA box is doubly underlined.

(D) Alignment between the amino-terminal half of the protein encoded by the candidate AP3/PI target gene and known NAC proteins mentioned in the text. Boldfaced letters represent amino acids identical in all six sequences (upper case) or in at least four sequences (lower case). Homology among the proteins was not significant beyond the point shown here.

Meyerowitz, 1996a), AP3-GR activated by dexamethasone could not replace sepals with petaloid organs unless *35S::PI* was also present. Flowers homozygous for *ap3-3* and *ag-3* carrying *35S::AP3-GR* and *35S::PI* formed only sepals in the absence of steroid; with dexamethasone treatment, all organs were petaloid (Figures 2C and 2F). Scanning electron microscopy confirmed that the epidermal cells of these petaloid organs had characteristics intermediate between those of sepal and petal epidermis (Figure 2J.) Thus, the AP3-GR fusion provided tight control of AP3 function, allowing induction of the complete program of stamen development and at least part of the program for petal formation, if PI was also expressed.

Identification of a mRNA Activated by AP3-GR

To identify mRNAs affected by activation of AP3 function, flowers of *ap3-3; ag-3; 35S::PI; 35S::AP3-GR* were treated for 6 hr with dexamethasone combined with cycloheximide or with cycloheximide alone, and mRNA populations were compared by differential display. Inhibition of protein synthesis by cycloheximide was confirmed by inhibition of GUS protein accumulation in *ap3-3; ag-3* flowers containing the *GUS* reporter gene directed by a heat shock–inducible promoter (data not shown). Out of several thousand mRNAs represented in the differential display, three cDNA species were isolated, corresponding to mRNAs consistently up-regulated by steroid treatment. The first candidate showed no homology to known sequences; a second candidate was homologous to *pumilio*, which participates in translational control of *hunchback* during posterior patterning in *Drosophila* (Barker et al., 1992) (data not shown). For the third candidate, which is described here in detail, overlapping cDNA fragments were isolated four times independently during this screen (Figure 3C). When used as a probe in Southern blots at high or low stringency, the longest partial cDNA detected only one genomic fragment (data not shown). RNA blot analysis showed that the corresponding 1.3 kb mRNA was more abundant in wild-type than in *ap3-3* mutant flowers, suggesting activation by *AP3* (Figure 3B).

Longer cDNAs and genomic sequences that included the partial cDNA sequence isolated by differential display were cloned. The longest cDNA [1.1 kb without poly(A) tail] was close to full length and encoded a 268–amino acid polypeptide (Figure 3C). The N-terminal half of the predicted protein contained a NAC domain (Figure 3D), named after the founding members of this gene family (*NAM, ATAF1–2, CUC2*; Souer et al., 1996; Aida et al., 1997). Inspection of the upstream genomic sequence and alignment with other NAC proteins indicated that the longest cDNA contained a complete coding sequence.

Both *NAM* (*No Apical Meristem,* from petunia) and *CUC2* (*CUP-SHAPED COTYLEDONS 2* from *Arabidopsis*) have developmental roles in the establishment of the shoot apical meristem and for separation of cotyledons and floral organs (Souer et al., 1996; Aida et al., 1997). Another *NAC* gene (*SENESCENCE UP-REGULATED 5*) has been identified in the tomato as a gene

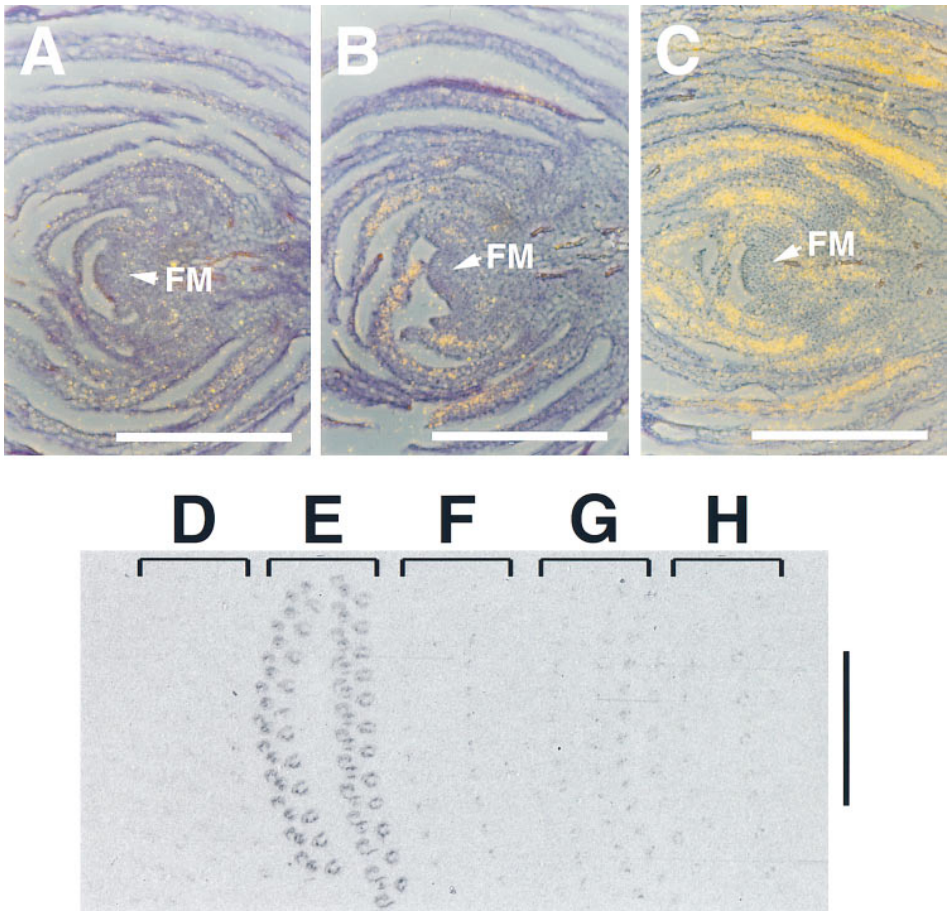

Figure 4. In situ Hybridization of *NAP* mRNA Induced by Steroid-Activated AP3-GR

All plants were *ap3-3; ag-3* and contained both *35S::AP3-GR* and *35S::PI* (see Figure 2C) unless stated otherwise. Flowers were soaked with 25 μl 0.015% Silwet L-77, 0.1% ethanol; when indicated, 10 μM cycloheximide and 5 μM dexamethasone were also present. Six hours later, flowers were fixed for in situ hybridization (performed as described in Experimental Procedures). (A–C), bar = 250 μm; (D–H), bar = 10 mm. (A–C) Longitudinal sections of individual flowers, showing the floral meristem (FM), surrounded by concentric layers of sepals of increasing age. Silver grains revealing hybridization of the ^{35}S-labeled probe appear yellow due to the filter used in the dark-field exposure, superimposed on the bright field image of the tissues. (A) untreated flower; (B) flower treated with cycloheximide alone; (C) flower treated with both cycloheximide and dexamethasone. (D–H) serial sections on a single microscope slide exposed to X-ray film; each of the exposures arranged in vertical rows corresponds to a single section as shown in (A–C). (D) flowers treated with cycloheximide alone; (E) flowers treated with cycloheximide and dexamethasone; (F) dexamethasone and cycloheximide-treated flowers, which lacked *35S::AP3-GR*; (G) flowers containing *35S::AP3-GR* but lacking *35S::PI*, treated with cycloheximide alone; (H) flowers containing *35S::AP3-GR* but lacking *35S::PI*, treated with both cycloheximide and dexamethasone.

whose expression in leaves peaks during senescence (John et al., 1997). Searching the databases for sequences encoding NAC domains revealed a large family in *Arabidopsis*, with at least 22 members with a maximum of 86% nucleotide sequence identity within the NAC region. As noted before, no homologous sequences were found in organisms other than plants (Souer et al., 1996; Aida et al., 1997). Based on its homology to *NAC* genes and on further evidence of regulation by *AP3/PI* (see below), the gene identified by differential display was named *NAP (NAC-LIKE, ACTIVATED BY AP3/PI)*.

NAP Activation Depends on Both AP3-GR and PI

In situ hybridization was performed to confirm that the candidate AP3/PI target gene was up-regulated by steroid-activated AP3-GR (Figure 4). Individual flowers of *ap3-3; ag-3; 35S::PI; 35S::AP3-GR* were treated with solution containing or lacking dexamethasone, in each case combined or not with cycloheximide. For all treatments, sense probe or vector sequences always revealed a low, uniform background signal (data not shown). The expression revealed by *NAP* antisense probe after mock treatment (Figure 4A) was indistinguishable from untreated *ap3-3; ag-3* flowers (Figure 5H); low and uniform expression was detected throughout sepals and increased in mature flowers. Treatment with cycloheximide alone modestly increased *NAP* mRNA levels, mostly in young sepals (Figure 4B). In contrast, treatment with cycloheximide combined with dexamethasone caused a large increase in expression, both in young and mature organs (Figure 4C). Similar results were obtained if dexamethasone was applied together with cycloheximide or with protein synthesis preinhibited by application of cycloheximide 90 min be-

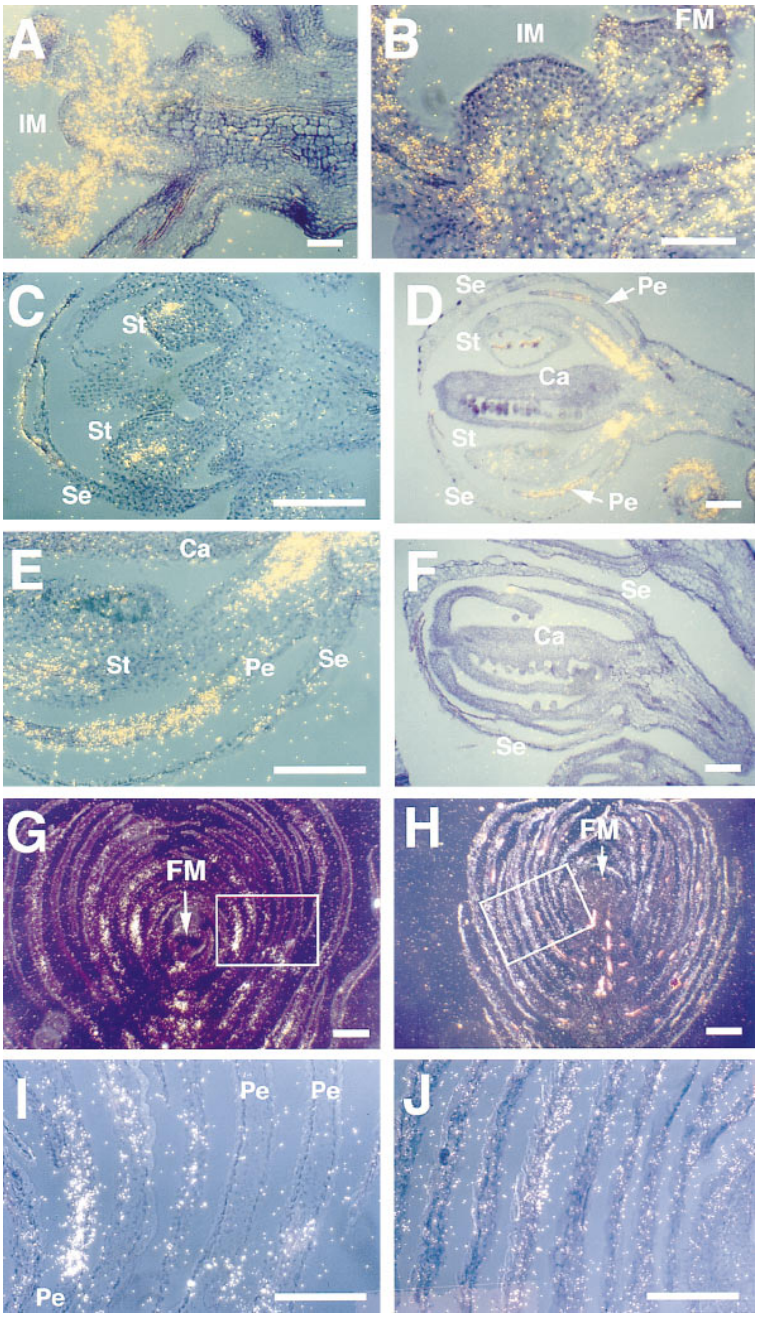

Figure 5. *NAP* Expression Pattern

In situ hybridization was performed as described in Experimental Procedures. Bar = 100 μm.

(A) Longitudinal section of the apex of a wild-type inflorescence bearing several fertilized flowers (not included in the section), showing expression below the inflorescence meristem (IM) in young flowers but not in more mature tissues. (B) Higher magnification of (A), showing little expression in the meristems of flowers (FM) and inflorescence (IM). (C) Longitudinal section of a wild-type stage-8 flowers, showing early expression in stamens (St); expression in stamens was more clearly seen in flowers from young inflorescences when expression in sepals (Se) and pedicels was lower. (D) Longitudinal section of a wild-type stage-10 flower, showing expression in petals (Pe) and stamens (St); sepals (Se) and carpels (Ca) are also indicated. (E) Expression in stage-10 flowers at higher magnification. (F) Longitudinal section of *ap3-3* flower at a stage comparable to the flower shown in (D). (G) Longitudinal section of *ag-3* flower, with the floral meristem (FM) surrounded by petals and sepals of increasing age; the area within the rectangle is shown at higher magnification in (I); Pe indicates petals, which are recognizable by their characteristic epidermal cells. (H) Longitudinal section of *ag-3; ap3-3* flower with sepals of increasing age surrounding the floral meristem (FM); the rectangle corresponds to (J).

fore addition of the steroid (data not shown). In the absence of cycloheximide, dexamethasone treatment for 6 hr did not cause a change in *NAP* mRNA levels that could be detected clearly by in situ hybridization (data not shown).

Steroid treatment had no effect on *NAP* expression in flowers lacking *35S::AP3-GR* (compare Figures 4E and 4F), showing that dexamethasone did not activate *NAP* directly. In flowers containing *35S::AP3-GR* but lacking *35S::PI*, steroid treatment also failed to activate *NAP* (compare Figures 4E–4H). Dependence on PI for activation strengthened the evidence that the mRNA upregulated by steroid-activated AP3-GR corresponds to a true *AP3/PI* target gene.

NAP Expression Pattern

Additional *in situ* hybridization was performed to test if *NAP* was expressed within the domain of *AP3/PI* activity. In vegetative tissues, no signal was detected above the low and uniform background also observed with sense probe (data not shown). In wild-type plants, the earliest *NAP* expression was detected below the inflorescence meristem and in the pedicels and developing sepals of young flowers (up to stage 8, defined by Smyth et al., 1990) (Figures 5A and 5B). Expression in the pedicel and sepals of young flowers was reduced as they matured and was replaced by expression in stamens, initially in what appeared to be the developing connective (Figure 5C) and later at the base of the stamen filaments (Figures

5D and 5E). *NAP* was also expressed in the distal part of the petal blade and along the middle vein as petals reached the length of the lateral stamens (stage 10; Figures 5D and 5E). In older petals, the hybridization signal increased at the base but was diminished or patchy on the expanding blades. In mature flowers, signal was also detected in ovules (data not shown) and increased in sepals (to levels similar to those seen in Figures 5H and 5J). Expression below the inflorescence meristem in petals and at the base of stamens was weaker just after bolting and increased in more mature inflorescences containing several fertilized flowers.

Transient *NAP* expression in petals was seen more clearly in *ag-3* flowers, which contain numerous petals at different stages of development. Expression in petal blades peaked at approximately the same stage as in wild-type flowers (Figures 5G and 5I). In contrast, *ap3-3; ag-3* flowers showed no distinct peak of *NAP* expression during sepal growth (Figures 5H and 5J).

In *ap3-3* mutant flowers at a stage comparable to the flower shown in Figure 5D, expression in the second and third whorls was low and uniform (Figure 5F), confirming that *NAP* expression did not simply reflect the position of organs within the flower, but it instead depended on the organ identities specified by *AP3/PI*. The increased expression in sepals and ovules of mature flowers was unaffected in *ap3-3* mutants (data not shown). Surprisingly, however, expression near the inflorescence meristem was also much reduced in *ap3-3* mutants (data not shown), even though *AP3* mRNA is not detectable in that region. Expression near the meristem was similarly reduced in *ag-3* mutants (data not shown). Common features of *ap3-3* and *ag-3* mutants are infertility and lack of stamens. Together with the gradual increase of *NAP* expression in wild-type inflorescences, these results suggest that *NAP* expression is increased by a diffusible signal produced by stamens, fertilized flowers, or developing siliques.

The results described above showed that not all aspects of *NAP* expression coincided with *AP3/PI* activity. Expression in petals and stamens, however, placed *NAP* within developmental programs under *AP3/PI* control. *NAP* mRNA accumulated in these organs relatively late in development and in a subset of their cells, implying that additional factors must limit the ability of *AP3/PI* to increase *NAP* expression.

Functional Analysis of *NAP*

NAP was mapped on chromosome 1, three map units distal to *APETALA1*. The only floral mutant known to map nearby is *pin-formed* (*pin1*; Bennett et al., 1995). Sequencing of the complete *NAP* coding region in two different *pin1* mutant alleles (*pin1-3, pin1-5*) revealed no mutations (data not shown). This, in addition to the different phenotype caused by antisense inhibition of *NAP* (see below), makes it unlikely that *NAP* and *PIN1* are the same gene. PCR screening of 6000 T-DNA-tagged *Arabidopsis* lines (McKinney et al., 1995; DNA pools from the Arabidopsis Stock Center, Ohio) failed to reveal insertions in *NAP*.

Because no *NAP* mutants were available, NAP function was studied in transgenic plants. For ubiquitous NAP expression, plants were transformed with the complete *NAP* coding sequence directed by the 35S promoter (*35S::NAP*). Overexpression was confirmed by

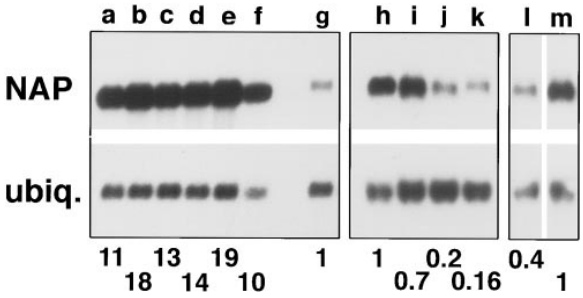

Figure 6. *NAP* mRNA Levels in *35S::NAP* and *35S::antiNAP* Plants

(Top) RNA gel blot of 2 μg poly(A)$^+$ RNA from inflorescences probed with *NAP* 3′ sequences (lanes a–g) or *NAP* 5′ sequences (lanes h–m). The same blots were reprobed with ubiquitin probe (bottom). Each boxed set of lanes corresponds to one separate experiment. The ratio of ubiquitin to NAP signals in wild-type control varies between experiments because blots were exposed for different times. Numbers below each lane indicate the level of *NAP* mRNA relative to wild-type after correction for loading, based on the ubiquitin controls. (lanes g, h, and m) wild-type; (lanes a–f) *35S::NAP*, independent lines. (lanes i–l) *35S::antiNAP*, independent lines.

RNA blot (Figure 6, lanes a–f); no lines were found with *NAP* cosuppression. Vegetative development of *35S::NAP* plants was indistinguishable from wild-type, but floral organs were affected, particularly petals and stamens (Figures 7A–7C). The most severe phenotype was seen in three independent lines, in which most flowers had very short petals and stamens (Figures 7A and 7C); the latter produced normal and functional pollen, but the flowers were female-sterile. In six intermediate lines, petals were still short, but stamens were nearly normal (Figure 7B). These lines showed a phenotypic gradient: early arising flowers had shorter petals than late flowers. Four additional lines had a mild phenotype with normal stamens but slightly shorter petals, which curled laterally and did not bend away from the gynoecium as in mature wild-type flowers. In the *ap3-3* mutant background, severe *35S::NAP* flowers had all floral organs slightly shorter; there was no discernible rescue of petal or stamen characteristics (data not shown).

Constitutive NAP expression affected organ growth by inhibiting cell expansion rather than proliferation. In stage-11 flowers, the sizes of petals and of their epidermal cells were similar in severe *35S::NAP* and wild-type flowers (Figures 7D and 7G). After stage 11, wild-type petals grow rapidly due to cell expansion, which was inhibited in *35S::NAP* flowers (Figures 7E, 7F, 7H, and 7I). Similarly, cells were shorter in stamen filaments of mature *35S::NAP* than in mature wild-type flowers (data not shown). In contrast, hypocotyl elongation was normal in etiolated seedlings of severe *35S::NAP* lines (data not shown), indicating that ubiquitous NAP expression affected cell expansion specifically in flowers.

Antisense inhibition of *NAP* was also tested. To avoid affecting expression of homologous genes, only the nonconserved part of the *NAP* coding sequence (between primers LH-AP4 and LH-T_{12}G in Figure 3C) was expressed in antisense orientation controlled by the 35S promoter (*35S::antiNAP*). Four independent lines, each segregating a single transgenic locus, displayed a subtle but characteristic phenotype, which was reproduced in subsequent generations; in the first 2–4 flowers of both

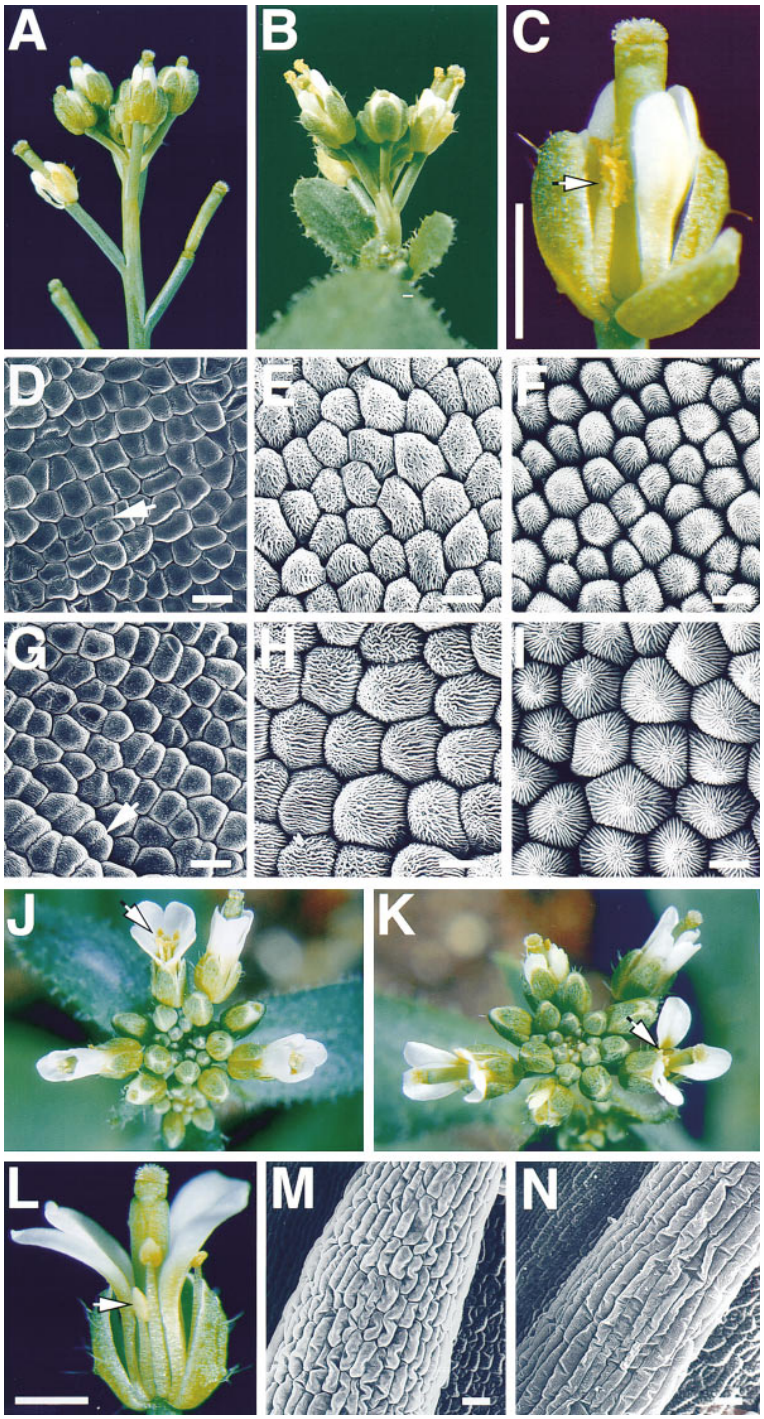

Figure 7. Phenotype of *35S::NAP* and *35S::antiNAP* Flowers

Bar = 1 mm (C and L), 5 μm (D–I), and 20 μm (M and N). (A and B) inflorescences of severe (A) and intermediate (B) *35S::NAP* lines, (corresponding to lanes b and d, respectively, on Figure 6). (C) severe *35S::NAP* line, single flower; arrow indicates short stamen. (D–I) Scanning electron microscopy view of petal epidermal cells in severe *35S::NAP* line (D–F) or wild-type flowers (G–I). (D and G): stage-11 flowers, abaxial surface; note the high frequency of dividing cells, some of which are indicated by arrows. (E and H) mature flowers, abaxial surface. (F and I): mature flowers, adaxial surface. (J) wild-type L-*er* inflorescence; the arrow points to mature stamens, which protrude above the gynoecium. (K) *35S::antiNAP* inflorescence (line corresponding to lane j in Figure 6) shortly after bolting; the arrow points to the short stamens. (L) higher magnification of a flower similar to those in (K) with one petal removed to expose the short stamens (arrow). (M and N) scanning electron micrographs of epidermal cells in stamen filaments of a mature *35S::antiNAP* flower (M) or mature wild-type flower (N).

the main and lateral inflorescences, stamens were short and their anthers often did not dehisce (Figures 7J–7L). Surprisingly, *35S::antiNAP* stamens had reduced cell elongation (Figure 7M), similar to *35S::NAP* stamens. One explanation for this similar effect is that a pulse of NAP expression is required for cell expansion (see Discussion).

RNA blot analysis confirmed that total *NAP* expression was reduced by 60%–80% in three of the *35S::antiNAP* lines (Figure 6, lanes j–l). One line, however (line 1, lane i), had nearly normal levels of *NAP* mRNA. There is evidence that antisense RNA acts not only by destabilizing the target mRNA but also by inhibiting its translation (Cornelissen and Vanderwiele, 1989); antibodies against NAP will be required to test whether NAP protein levels are reduced in line 1.

Discussion

An Immediate *AP3/PI* Target Gene

Several lines of evidence together indicate that *NAP* is activated by *AP3/PI*. First, activated AP3-GR, which mimics AP3 function, up-regulated *NAP* in a PI-dependent manner. Activation of *NAP* by AP3-GR with protein

synthesis blocked indicated that *NAP* is an immediate target of *AP3/PI*; a similar approach has been used previously to demonstrate immediate activation of *cdc25* by c-Myc in mouse LM3 cells (Galaktionov et al., 1996). Formally, it cannot be excluded that the effect was on stability of the *NAP* message rather than on transcription, but we favor the hypothesis of transcriptional activation based on the fact that AP3 and PI belong to a well-studied family of transcription factors and on their binding to specific DNA sequences in vitro. The strong up-regulation seen in Figure 4C not only tolerated but actually required cycloheximide treatment. Two reasons, singularly or combined, may explain both the superinduction by steroid in the presence of cycloheximide and the weaker induction by cycloheximide alone: decay of a preexisting, short-lived inhibitor of *NAP* mRNA accumulation; or blockage of a negative feedback loop that normally would cause *NAP* expression to be transient, analogous to the mechanism used to cause transient expression of immediate targets of ecdysone activation in *Drosophila* (Thummel et al., 1990).

Second, the expression pattern of *NAP* is consistent with AP3/PI being one of its regulators. *AP3/PI* mRNAs are detectable initially in stamen and petal primordia of stage-3 flowers, then remain at high levels throughout the development of these organs (Jack et al., 1992; Goto and Meyerowitz, 1994). Strong *NAP* expression was detected within the domain of *AP3/PI* activity and was absent in *ap3-3* mutants. *NAP* expression in petals was expected, based on its activation by AP3-GR, during a switch from sepal to petaloid gene expression. *NAP* activity in stamens, although initially surprising, placed *NAP* in a different developmental program, which is also controlled by *AP3/PI.* However, part of the *NAP* expression pattern either did not depend on *AP3*, as seen in ovules or sepals of mature flowers, or did not coincide with *AP3/PI* expression (near inflorescence tips). In the latter case, expression may be increased by a diffusible signal produced in stamens or fertilized flowers; plant growth regulators would be obvious candidates for such a signal.

Third, the stamen defects seen in *35S::antiNAP* plants showed that *NAP* function is important for a developmental program controlled by *AP3/PI*. The high levels of *NAP* mRNA in wild-type stamens, combined with the stamen defects caused by a partial loss of *NAP* expression, suggested that stamens are the organs requiring the highest levels of *NAP* activity for normal development. The fact that the stamen defect was seen in the first few flowers of *35S::antiNAP* plants may reflect the lower level of *NAP* expression observed in young inflorescences.

Direct association between a homeotic protein and target DNA sequences has been proposed as one of the criteria that must be satisfied by candidate homeotic target genes (Andrew and Scott, 1992). AP3/PI bind in vitro to a CArG–box-like sequence in the first intron of *NAP* (data not shown), but the significance of this kind of evidence in our case is doubtful for two reasons. First, there is considerable overlap of in vitro DNA binding specificity between different MADS proteins (Riechmann et al., 1996b). Second, chimeric MADS proteins with altered DNA-binding domains had no noticeable change in their homeotic activity, even when in vitro binding specificity was clearly altered (Krizek and Meyerowitz, 1996b; Riechmann and Meyerowitz, 1997a). In vivo footprinting combined with AP3-GR activation may be a more incisive approach to study how AP3/PI (and other factors) interact with *NAP*.

Previously identified genes have been proposed to be controlled by floral homeotic genes, mainly based on the changes in their expression patterns caused by homeotic mutations (reviewed by Riechmann and Meyerowitz, 1997b). *NAP*, however, is the first case in which there is strong evidence of immediate regulation by a plant homeotic gene.

Direct Activation of a Late Developmental Step by *AP3/PI*

Homeotic genes must control target genes not only spatially but also in a defined temporal pattern. Different models can be imagined to explain how time is measured during these developmental programs. A simple sequential gene activation with later steps independent of the initial regulators can be ruled out; one of the defining characteristics of homeotic selector genes is that their activity is required throughout the developmental programs they control (Garcia-Bellido, 1975), and this has also been verified for floral homeotic genes (Bowman et al., 1989; Zachgo et al., 1995). Time could still be set by sequential target gene activity, with target genes acting together with homeotic genes to select the next subordinate genes in the sequence. In this case, if a developmental program had to be redirected by artificially changing the homeotic selector at a late stage, the whole sequence would have to be recapitulated. Alternatively, a homeotic function could read time from clues independent of homeotic gene activity. These clues might have been maintained from the ancestral "ground state" developmental program, which is modified by homeotic genes. The ground state of floral organs in the absence of homeotic gene activity is leaflike (Bowman et al., 1989). The second model predicts that late activation of a homeotic function would directly select late target genes. These extreme models may also be combined, with some time clues being independent of homeotic function and others not. Experiments based on the phenotypic effects of late restoration of homeotic gene activity using temperature-sensitive alleles (Bowman et al., 1989; Zachgo et al., 1995) or transposon excision (Carpenter and Coen, 1991) cannot easily distinguish between these models, because phenotypic rescue after late activation does not exclude recapitulation of a regulatory cascade; furthermore, lack of phenotypic restoration does not imply lack of gene activation, especially for functions that require further cell divisions and growth to become visible.

The results described here eliminate the extreme model of sequential target gene activation. The screen for target genes was performed in flowers containing predominantly sepals in late stages of development. Correspondingly, the immediate target gene identified is expressed at late stages of development both in petals and stamens. Therefore, recapitulation is not necessary for activation of at least one of the late downstream

genes. Although the model based on time clues independent of homeotic gene function is not proven, it is supported here and must be considered in future screens for target genes: one of its corollaries is that identification of the earliest *AP3/PI* target genes will only be possible by using organs at early stages of development, regardless of the homeotic activities present.

NAP Function

One of the objectives of identifying target genes is to understand the link between homeotic gene activity and morphogenesis. Plants do not use cell migration and differential cell adhesion for development; their major morphogenetic tools are cell division coupled with regulated cell expansion (reviewed by Meyerowitz, 1997). In young organs, cell division is frequent. As organs mature, division is infrequent or stops completely, and growth occurs by cell expansion. In shoots and roots, these modes of growth are seen along the main axis with active cell division in the apical meristems, followed by transition zones and then elongation zones. Cells in transition areas may have special properties, being particularly sensitive to cues controlling cell division (Jacobs, 1997).

These distinct growth phases are also visible in petals and stamens. In petunia, for example, clonal analysis by transposon excision from a visible marker gene showed that the large increase in petal size at the end of flower development is due exclusively to cell expansion (Martin and Gerats, 1993). In petals and stamens, appropriate timing of cell expansion is essential to coordinate floral maturation with opening of flowers and fertilization.

To determine the precise function of *NAP* in flower development, a complete loss-of-function mutant must be isolated. The available data, however, suggest that *NAP* functions in the transition between active cell division and cell expansion. *NAP* is transiently expressed in cells while they are adjacent to inflorescence or floral meristems; later expression in petal blades occurs at the transition between stages of frequent cell division (see Figures 7D and 7G) and rapid cell expansion (Figures 7E, 7F, 7H, and 7I); in stamen filaments, *NAP* mRNA is also detected preceding cell elongation. The transient expression of *NAP* is consistent with its superinduction by cycloheximide, which, as discussed above, may be caused by obstruction of a negative feedback mechanism. Ubiquitous and continuous expression of NAP inhibited cell expansion. NAP, however, cannot be simply a negative regulator of cell expansion, because partial loss of its function also inhibited cell elongation in stamen filaments of early flowers. The results of misexpression are compatible with a transient function of NAP. In its absence, cells cannot shift to elongation mode; with sustained expression, the transition cannot be finished. Alternatively, a defined level of NAP may be necessary for proper cell elongation, but we consider this hypothesis weaker because it is indifferent to the expression pattern of *NAP* and to the possible negative feedback revealed by cycloheximide superinduction. Ubiquitous NAP expression had flower-specific effects, suggesting that floral cofactors are necessary for NAP stability or function.

Are Other NAC Genes Regulated by Floral Homeotic Genes?

The expression pattern of *NAP* implied that it is not exclusively an *AP3/PI* target gene. Similarly, in *Drosophila*, several homeotic target genes have other roles not modulated by homeotic genes; the expression patterns are further complicated in several cases where the same target genes are controlled by multiple homeotic genes (reviewed by Graba et al., 1997). The use of the same genes in different developmental programs seems to support the view that during evolution, "new" selector genes simply recruit already available genes and use them in different ways (Garcia-Bellido, 1975).

In many cases, however, functionally linked genes seem to have coevolved (Fryxell, 1996). Similar regulatory circuits, each evolving as a unit after duplication of an ancestral circuit, can also be used for different purposes in the same organism, as observed with the Toll-Dorsal/Cactus dorsoventral patterning and MyD88-Dif immune reponse pathways in *Drosophila* (reviewed by Fryxell, 1996). Therefore, it is possible that as homeotic genes diverged during evolution, at least some of the ancestral target genes may also have duplicated and specialized under different homeotic regulators.

Like MADS-box genes (see Münster et al., 1997, and references therein), *NAP* belongs to a large gene family in *Arabidopsis*, at least some of whose members are also expressed in flowers (since they were identified as ESTs from flower cDNA libraries). Both *NAM* and *CUC2* have floral functions that seem to be partially redundant and which might be covered by additional NAC family members (Souer et al., 1996; Aida et al., 1997), again suggesting that multiple NAC genes may function in flowers. It will be interesting to see if any of the other genes in the family are also controlled by different floral homeotic genes, which would suggest coevolution of MADS-box and NAC genes. Alternatively, recruitment of a NAC gene may have made a unique contribution to the genetic programs controlled by *AP3/PI*.

Experimental Procedures

Plasmid Constructions

All cloning procedures were standard (Sambrook et al., 1989). To create the AP3-GR fusion, the *AP3* coding sequence (from pF730, Jack et al., 1992) was polymerase chain reaction (PCR)-mutagenized to create a SnaBI site replacing the stop codon. The rat GR fragment was inserted into the SnaBI site as a Klenow-filled BamHI-EcoICRI fragment from pBI-ΔGR (gift from Allan Lloyd, Stanford University). The protein sequence was (AP3 aa 1–232)-Tyr-Asp-Pro-(GR aa 508–795). AP3-GR was then cloned in pCGN18 (Krizek and Meyerowitz, 1996a) to create p35S::AP3-GR.

p35S::NAP had the complete NAP coding sequence (see below) in pCGN18. To create p35S::antiNAP, the differential display product LH-AP4/LH-T12G (Figure 3A) was cloned in antisense orientation in pCGN18. p35S::PI has been described (Krizek and Meyerowitz, 1996a).

Plant Transformation and Growth

p35S::AP3-GR, p35S::PI, p35S::NAP, and p35S::antiNAP were transformed into *Agrobacterium tumefaciens* strain ASE, which was then used to transform *Arabidopsis thaliana*, ecotype Landsberg *erecta* (L-*er*), by vacuum infiltration (Bechtold et al., 1993). Bleach-sterilized seeds were plated on MS medium with 50 μg/ml kanamycin, vernalized at 4°C for 4 days, and germinated for 10 days at 22°C under

continuous 600-foot candles of cool-white fluorescent light. Kanamycin-resistant plants were potted on a mixture of 4:3:2 (soil:vermiculite:perlite) and returned to the conditions used for germination.

Construction of Plant Lines

The *35S::PI* line chosen for further studies had a single transgenic locus and displayed the typical phenotype described by Krizek and Meyerowitz (1996a). The *35S::AP3-GR* line chosen for construction of strains caused the strongest rescue of *ap3-3* and had a single transgenic locus.

Plants were manually cross-pollinated to combine the transgenes and the *ap3–3* and *ag-3* mutations. When necessary, presence of the transgenes was monitored by PCR from leaf pieces (Klimyuk et al., 1993), using cDNA-specific primers for AP3 and PI; *ag-3*/$^{+}$ plants were identified by PCR primer–introduced restriction analysis (Jacobson and Moscovits, 1991).

Differential Display

Flowers were treated as described in Figure 2; six flowers were pooled for each sample. Tissues were ground in liquid nitrogen and RNA was extracted with Tri-Reagent (Molecular Research Center) according to the manufacturer's instructions. DNase I treatment and cDNA synthesis from 350 ng total RNA were as described (Reuber and Ausubel, 1995), except that the first 10 min of reverse transcription were at 25°C and the primer used was LH-T12N (5′TGC CGAAGCT$_{12}$N3′, N being G, C, or A). Amplification was as described (Zhao et al., 1995), except that ^{33}P-dATP (Amersham) was used, high-stringency annealing was at 55°C, and arbitrary primers were LH-AP (5′TGCCGAAGCTTN$_7$; N$_7$ was seven arbitrary bases, which for the primers in Figure 3A were LH-AP4, 5′CTGAGTG3′ and LH-AP15, 5′AACCACG3′). Gel analysis and cloning of cDNAs were as described by Reuber and Ausubel (1995).

Cloning of *NAP* cDNA and Genomic Sequences

The N-terminal *NAP* coding sequence was cloned from a floral cDNA library in lambda ZAPII (Stratagene). Primers containing suitable restriction sites were then designed (nap1: 5′GAAA*GGATCC*AAACA GTTCCTGTTCTATTAGATTG3′ and nap2: 5′ATTGT*TCTAGA*TTTCAC ATTCATATGGGAG3′) for high-fidelity amplification (using *Pfu* polymerase, Stratagene) of the complete *NAP* coding sequence from the cDNA samples used for differential display or from genomic DNA. Independent PCR products were cloned and sequenced to confirm absence of PCR-caused mutations. Genomic sequences upstream of nap1 and downstream of nap2 were cloned using the PromoterFinder kit (Clontech). Sequencing was done with the ABI Prism kit (Applied Biosystems) and the Applied Biosystems model 373 DNA Sequencer. For sequence analysis, the GCG package was used (Genetics Computer Group). Database searches (GenBank and TIGR) were performed using the BLAST program (Altschul et al., 1990).

RNA Blots

Total RNA was extracted from 0.1–0.2 g flowers, as described above, and poly(A)$^{+}$ RNA was prepared with the PolyATract kit (Promega); 2 μg samples were analyzed by standard RNA blot (Sambrook et al., 1989). Probes were generated by linear PCR amplification (Konat et al., 1994). 3′ *NAP* probe covered the sequence from primers LH-AP4 and LH-AP12A (Figure 3C); 5′ *NAP* probe covered nucleotides 15–374 of the longest cDNA indicated in Figure 3C. Ubiquitin template was amplified from L-*er* genomic DNA, using primers NUBQ (5′GGTGCTAAGAAGAGGAAGAAT3′) and CUBQ (5′CTCCTTCTTTC TGGTAAACGT3′); CUBQ was used for probe synthesis. A PhosphorImager (Molecular Dynamics) was used for quantitative analysis.

RFLP Mapping

NAP was mapped based on an EcoRI polymorphism between ecotypes Landsberg and Columbia, according to Chang et al. (1988). The 3′ *NAP* probe for Southern blots was the same used for RNA blots.

In Situ Hybridization

Tissues were vacuum-infiltrated with PBS containing 4% paraformaldehyde, 0.1% Triton X-100, 0.1% Tween 20, then fixed in this solution overnight at 4°C. Other procedures were as described (Drews et al., 1991). The template for riboprobe synthesis was constructed by cloning a HindIII-XbaI fragment corresponding to the 3′ half of the *NAP* cDNA into pSPT19 (Boehringer Mannheim); polylinker sequences were then removed by EcoRI/XbaI digestion, filling in with Klenow and religation.

Scanning Electron Microscopy

Samples were fixed, dried, coated, and dissected as described (Bowman et al., 1989, 1991).

Acknowledgments

We are grateful to L. Medrano for help with restriction fragment length polymorphism mapping, T. Jack for pF730, B. Krizek for the 35S::PI plasmid, A. Lloyd for pBI-ΔGR, J. L. Riechmann for PromoterFinder libraries, M. Callahan for help with PCR genotyping, P. Koen for help with scanning electron microscopy, and to K. Baker, X. Chen, C. -F. Chuang, J. Fletcher, J. Hua, T. Ito, C. Ohno, J. L. Riechmann, K. Roberg, D. Wagner, R. Williams, and E. Ziegelhoffer for critical reading of the manuscript. R. W. M. S. was supported by a long-term fellowship (LT-12/95) from the Human Frontier Science Program, and work in E. M. M.'s laboratory is supported by National Science Foundation grant MCB-9603821.

Received November 3, 1997; revised November 24, 1997.

References

Aida, M., Ishida, T., Fukaki, H., Fujisawa, H., and Tasaka, M. (1997). Genes involved in organ separation in Arabidopsis: an analysis of the *cup-shaped cotyledon* mutant. Plant Cell *9*, 841–857.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403–410.

Andrew, D.J., and Scott, M.P. (1992). Downstream of the homeotic genes. New Biol. *4*, 5–15.

Aoyama, T., and Chua, N.-H. (1997). A glucocorticoid-mediated transcriptional induction system in transgenic plants. Plant J. *11*, 605–612.

Barker, D.D., Wang, C., Moore, J., Dickinson, L.K., and Lehmann, R. (1992). Pumilio is essential for function but not for distribution of the Drosophila abdominal determinant Nanos. Genes Dev. *6*, 2312–2326.

Bechtold, N., Ellis, J., and Pelletier, G. (1993). In planta *Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. CR Acad. Sci. (Paris) *316*, 1194–1199.

Bennett, S.R.M., Alvarez, J., Bossinger, G., and Smyth, D.R. (1995). Morphogenesis in *pinoid* mutants of *Arabidopsis thaliana*. Plant J. *8*, 505–520.

Bowman, J.L., Smyth, D.R., and Meyerowitz, E.M. (1989). Genes directing flower development in *Arabidopsis*. Plant Cell *1*, 37–52.

Bowman, J.L., Smyth, D.R., and Meyerowitz, E.M. (1991). Genetic interactions among floral homeoic genes of *Arabidopsis*. Development *112*, 1–20.

Carpenter, R., and Coen, E.S. (1991). Floral homeotic mutations produced by transposon-mutagenesis in *Antirrhinum majus*. Genes Dev. *4* 1483–1493.

Chang, C., Bowman, J.L., DeJohn, A., Lander, E.S., and Meyerowitz, E.M. (1988). Restriction fragment length polymorphism linkage map for *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA *85*, 6856–6860.

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

Cornelissen, M., and Vanderwiele, M. (1989). Both RNA level and translation efficiency are reduced by anti-sense RNA in transgenic tobacco. Nucl. Acids Res. *17*, 833–843.

Dalman, F.C., Scherrer, L.C., Taylor, L.P., Akil, H., and Pratt, W.B. (1991). Localization of the 90 kDa heat shock protein-binding site within the hormone-binding domain of the glucocorticoid receptor by peptide competition. J. Biol. Chem. *266*, 3482–3490.

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.

Fryxell, K.J. (1996). The coevolution of gene family trees. Trends Genet. *12*, 364–369.

Galaktionov, K., Chen, X., and Beach, D. (1996). Cdc25 cell-cycle phosphatase as a target of c-*myc*. Nature *382*, 511–517.

Garcia-Bellido, A. (1975). Genetic control of wing disc development in *Drosophila*. In Cell Patterning, Ciba Foundation Symp. *29*, 161–178.

Goto, K., and Meyerowitz, E.M. (1994). Function and regulation of the *Arabidopsis* homeotic gene *PISTILLATA*. Genes Dev. *8*, 1548–1560.

Graba, Y., Aragnol, D., and Pradel, J. (1997). Drosophila Hox complex downstream targets and the function of homeotic genes. Bioessays *19*, 379–388.

Hill, J.P., and Lord, E.M. (1989). Floral development in *Arabidopsis thaliana*: a comparison of the wild-type and the homeotic pistillata mutant. Can. J. Bot. *67*, 2922–2936.

Jack, T., Brockman, L.L., and Meyerowitz, E.M. (1992). The homeotic gene *APETALA 3* of *Arabidopsis thaliana* encodes a MADS box and is expressed in petals and stamens. Cell *68*, 683–687.

Jack, T., Fox, G.L., and Meyerowitz, E.M. (1994). *Arabidopsis* homeotic gene *APETALA 3* ectopic expression: transcriptional and posttranscriptional regulation determine floral organ identity. Cell *76*, 703–716.

Jacobs, T. (1997). Why do plant cells divide? Plant Cell *9*, 1021–1029.

Jacobson, D.R., and Moscovits, T. (1991). Rapid, nonradioactive screening for activating *ras* oncogene mutations using PCR primer-introduced restriction analysis (PCR-PIRA). PCR Meth. Appl. *1*, 146–148.

John, I., Hackett, R., Cooper, W., Drake, R., Farrel, A., and Grierson, D. (1997). Cloning and characterization of tomato leaf senescence-related cDNAs. Plant Mol. Biol. *33*, 641–651.

Klimyuk, V.I., Carroll, B.J., Thomas, C.M., and Jones, J.D.G. (1993). Alkali treatment for rapid preparation of plant material for reliable PCR analysis. Plant J. *3*, 493–494.

Konat, G.W., Laszkiewicz, I., Grubinska, B., and Wiggins, R.C. (1994). Generation of labeled probes by PCR. In PCR Technology: Current Innovations, H.G. Griffin and A.M. Griffin, eds., (Boca Raton: CRC).

Krizek, B.A., and Meyerowitz, E.M. (1996a). The *Arabidopsis* homeotic genes *APETALA 3* and *PISTILLATA* are sufficient to provide the B class organ identity function. Development *122*, 11–22.

Krizek, B.A., and Meyerowitz, E.M. (1996b). Mapping the protein regions responsible for the functional specificities of the *Arabidopsis* MADS domain organ identity proteins. Proc. Natl. Acad. Sci. USA *93*, 4063–4070.

Krumlauf, R. (1994). *Hox* genes in vertebrate development. Cell *78*, 191–201.

Lawrence, P.A., and Morata, G. (1994). Homeobox genes: their function in *Drosophila* segmentation and pattern formation. Cell *78*, 181–189.

Liang, P., and Pardee, A.B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science *257*, 967–971.

Lloyd, A.M., Schena, M., Walbot, V., and Davis, R. (1994). Epidermal cell fate determination in *Arabidopsis*: patterns defined by a steroid-inducible regulator. Science *266*, 436–439.

Martin, C., and Gerats, T. (1993). Control of pigment biosynthesis genes during petal development. Plant Cell *5*, 1253–1264.

McKinney, E.C., Ali, N., Traut, A., Feldmann, K., Belostotsky, D.A., McDowell, J.M., and Meagher, R.B. (1995). Sequence-based identification of T-DNA insertion mutations in *Arabidopsis*: actin mutants *act2–1* and *act4–1*. Plant J. *8*, 613–622.

Meyerowitz, E.M. (1997). Genetic control of cell division patterns in developing plants. Cell *88*, 299–308.

Münster, T., Pahnke, J., Di Rosa, A., Kim, J.T., Martin, W., Saedler, H., and Theissen, G. (1997). Floral homeotic genes were recruited from homologous MADS-box genes preexisting in the common ancestor of ferns and seed plants. Proc. Natl. Acad. Sci. USA *94*, 2415–2420.

Reuber, T.L., and Ausubel, F.M. (1995). Differential mRNA display. Meth. Cell Biol. *49*, 431–440.

Riechmann, J.L., and Meyerowitz, E.M. (1997a). Determination of floral organ identity by *Arabidopsis* MADS domain homeotic proteins AP1, AP3, PI and AG is independent of their DNA-binding specificity. Mol. Biol. Cell *8*, 1243–1259.

Riechmann, J.L., and Meyerowitz, E.M. (1997b). MADS domain proteins in plant development. Biol. Chem. *378*, 1079–1101.

Riechmann, J.L., Krizek, B.A., and Meyerowitz, E.M. (1996a). Dimerization specificity of *Arabidopsis* MADS domain homeotic proteins APETALA1, APETALA3, PISTILLATA and AGAMOUS. Proc. Natl. Acad, Sci. USA *93*, 4793–4798.

Riechmann, J.L., Wang, M., and Meyerowitz, E.M. (1996b). DNA-binding properties of *Arabidopsis* MADS domain homeotic proteins APETALA1, APETALA3, PISTILLATA and AGAMOUS. Nucl. Acids Res. *24*, 3134–3141.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Simon, R., Igeño, M.I., and Coupland, G. (1996). Activation of floral meristem identity genes in *Arabidopsis*. Nature *384*, 59–62.

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

Souer, E., van Houwelingen, A., Kloos, D., Mol, J., and Koes, R. (1996). The *No Apical Meristem* gene of Petunia is required for pattern formation in embryos and flowers and is expressed at meristem and primordia boundaries. Cell *85*, 159–170.

Thummel, C.S., Burtis, K.C., and Hogness, D.S. (1990). Spatial and temporal patterns of E74 transcription during *Drosophila* development. Cell *61*, 101–111.

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

Yanofsky, M.F. (1995). Floral meristems to floral organs: genes controlling early events in Arabidopsis flower development. Annu. Rev. Plant Physiol. Plant Mol. Biol. *46*, 167–188.

Zachgo, S, Silva, E.A., Motte, P., Tröbner, W., Saedler, H., and Schwarz-Sommer, Z. (1995). Functional analysis of the *Antirrhinum* homeotic *DEFICIENS* gene in vivo and in vitro using a temperature-sensitive mutant. Development *121*, 2861–2875.

Zhao, S., Ooi, S.L., and Pardee, A.B. (1995). New primer strategy improves precision of differential display. Biotechniques *18*, 842–850.

GenBank Accession Number

The GenBank accession number for the new gene sequence reported in this paper is AJ222713.