Control of Arabidopsis Flower and Seed Development by the Homeotic Gene APETALA2
Author(s): K. Diane Jofuku, Bart G. W. den Boer, Marc Van Montagu and Jack K. Okamuro
Source: *The Plant Cell*, Vol. 6, No. 9 (Sep., 1994), pp. 1211-1225
Published by: American Society of Plant Biologists (ASPB)
Stable URL: http://www.jstor.org/stable/3869820
Accessed: 17/07/2014 21:23

Your use of the JSTOR archive indicates your acceptance of the Terms & Conditions of Use, available at
http://www.jstor.org/page/info/about/policies/terms.jsp

JSTOR is a not-for-profit service that helps scholars, researchers, and students discover, use, and build upon a wide range of content in a trusted digital archive. We use information technology and tools to increase productivity and facilitate new forms of scholarship. For more information about JSTOR, please contact support@jstor.org.

American Society of Plant Biologists (ASPB) is collaborating with JSTOR to digitize, preserve and extend access to *The Plant Cell*.

http://www.jstor.org

This content downloaded from 164.67.109.135 on Thu, 17 Jul 2014 21:23:12 PM
All use subject to JSTOR Terms and Conditions

The Plant Cell, Vol. 6, 1211–1225, September 1994 © 1994 American Society of Plant Physiologists

RESEARCH ARTICLE

Control of Arabidopsis Flower and Seed Development by the Homeotic Gene *APETALA2*

K. Diane Jofuku,[a] Bart G. W. den Boer,[b] Marc Van Montagu,[b] and Jack K. Okamuro [a,1]

[a] Sinsheimer Laboratories, Department of Biology, University of California, Santa Cruz, California 95064
[b] Laboratorium voor Genetika, Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium

***APETALA2* (*AP2*) plays a central role in the establishment of the floral meristem, the specification of floral organ identity, and the regulation of floral homeotic gene expression in Arabidopsis. We show here that in addition to its functions during flower development, *AP2* activity is also required during seed development. We isolated the *AP2* gene and found that it encodes a putative nuclear protein that is distinguished by an essential 68-amino acid repeated motif, the AP2 domain. Consistent with its genetic functions, we determined that *AP2* is expressed at the RNA level in all four types of floral organs—sepals, petals, stamens, and carpels—and in developing ovules. Thus, *AP2* gene transcription does not appear to be spatially restricted by the floral homeotic gene *AGAMOUS* as predicted by previous studies. We also found that *AP2* is expressed at the RNA level in the inflorescence meristem and in nonfloral organs, including leaf and stem. Taken together, our results suggest that AP2 represents a new class of plant regulatory proteins that may play a general role in the control of Arabidopsis development.**

INTRODUCTION

The pattern of flower development is controlled by the floral meristem, a complex tissue whose cells give rise to the different organ systems of the flower. Genetic and molecular studies have defined a network of genes that control floral meristem identity and floral organ development in Arabidopsis, snapdragon, and other plant species (reviewed by Coen and Carpenter, 1993; Okamuro et al., 1993; van der Krol and Chua, 1993; Veit et al., 1993; Ma, 1994). In Arabidopsis, the floral homeotic gene *APETALA2* (*AP2*) controls three critical aspects of flower ontogeny—the establishment of the floral meristem (Irish and Sussex, 1990; Huala and Sussex, 1992; Bowman et al., 1993; Schultz and Haughn, 1993; Shannon and Meeks-Wagner, 1993), the specification of floral organ identity (Komaki et al., 1988; Bowman et al., 1989; Kunst et al., 1989), and the temporal and spatial regulation of floral homeotic gene expression (Bowman et al., 1991a; Drews et al., 1991a).

One early function of *AP2* during flower development is to promote the establishment of the floral meristem. *AP2* performs this function in cooperation with at least three other floral meristem genes, *APETALA1* (*AP1*), *LEAFY* (*LFY*), and *CAULIFLOWER* (*CAL*) (Irish and Sussex, 1990; Bowman, 1992; Huala and Sussex, 1992; Bowman et al., 1993; Schultz and Haughn, 1993; Shannon and Meeks-Wagner, 1993). A second function of *AP2* is to regulate floral organ development. In Arabidopsis, the floral meristem produces four concentric rings or whorls of floral organs—sepals, petals, stamens, and carpels. In weak, partial loss-of-function *ap2* mutants, sepals are homeotically transformed into leaves, and petals are transformed into pollen-producing stamenoid organs (Bowman et al., 1989, 1991b). By contrast, in strong *ap2* mutants, sepals are transformed into ovule-bearing carpels, petal development is suppressed, the number of stamens is reduced, and carpel fusion is often defective (Komaki et al., 1988; Kunst et al., 1989; Bowman et al., 1991b). Finally, the effects of *ap2* on floral organ development are in part a result of a third function of *AP2*, which is to directly or indirectly regulate the expression of several flower-specific homeotic regulatory genes (Bowman et al., 1991a; Drews et al., 1991a; Jack et al., 1992; Mandel et al., 1992a).

Clearly, *AP2* plays a critical role in the regulation of Arabidopsis flower development. Yet, little is known about how it carries out its functions at the cellular and molecular levels. A spatial and combinatorial model has been proposed to explain the role of *AP2* and other floral homeotic genes in the specification of floral organ identity (Haughn and Somerville, 1988; Bowman et al., 1991a; Coen and Meyerowitz, 1991; Drews et al., 1991b; Meyerowitz et al., 1991; Bowman et al., 1993; Coen and Carpenter, 1993). One central premise of this model is that *AP2* and a second floral homeotic gene *AGAMOUS* (*AG*) are mutually antagonistic genes. That is, *AP2* negatively regulates *AG* gene expression in sepals and petals, and conversely, *AG* negatively regulates *AP2* gene expression in stamens and

[1] To whom correspondence should be addressed.

This content downloaded from 164.67.109.135 on Thu, 17 Jul 2014 21:23:12 PM
All use subject to JSTOR Terms and Conditions

carpels. In situ hybridization analysis of *AG* gene expression in wild-type and *ap2* mutant flowers has demonstrated that *AP2* is indeed a negative regulator of *AG* expression (Bowman et al., 1991a; Drews et al., 1991a). However, it is not yet known how *AP2* controls *AG.* Nor is it known how *AG* influences *AP2* gene activity.

As a first step toward understanding how *AP2* functions in Arabidopsis, we isolated the *AP2* gene by T-DNA insertional mutagenesis. In this study, we show that *AP2* encodes a putative nuclear factor that bears no significant similarity to any known plant, fungal, or animal regulatory protein. Our analysis indicates that *AP2* gene activity and function are not restricted to or within developing flowers, suggesting that it may play a broader role in the regulation of Arabidopsis development than originally proposed.

RESULTS

Isolation of an *ap2* Insertion Mutant

We utilized T-DNA from Agrobacterium as an insertional mutagen to identify and isolate genes controlling flower formation in Arabidopsis (Van Lijsebettens et al., 1991). One transformed line, designated T10, segregated 3 to 1 for a flower mutant that phenotypically resembled many allelic forms of the floral homeotic mutant *ap2* (Koornneef et al., 1980; Komaki et al., 1988; Bowman et al., 1989, 1991b; Kunst et al., 1989). We tested and confirmed genetically that T10 and *ap2* are allelic (data not shown) and designated this mutant as *ap2-10.*

As with most severe *ap2* loss-of-function mutants, *ap2-10* affects the identity or development of all four types of floral organs. As shown in Figure 1A and depicted schematically in Figure 1B, a wild-type Arabidopsis flower contains four concentric whorls of floral organs consisting of four sepals, four petals, six stamens, and two carpels that are congenitally fused to form the pistil or gynoecium. By contrast, Figures 1C and 1D show that an *ap2-10* flower is characterized by the homeotic transformation of the medial sepals into chimeric sepaloid carpels. These organs are distinguished by the presence of stigmatic papillae and multiple ovules along the margin of each organ. The lateral sepals are either absent or develop into leaflike or filamentous organs (Figure 1C). Second-whorl organs, which normally develop into petals, are absent in *ap2-10.* Third-whorl stamens are reduced in number from six in wild type to between two and four organs in *ap2-10.* In addition, the distal tip of *ap2-10* anthers is often capped by a small patch of stigmatic papillae (Figure 1C), indicating a partial transformation of stamens to carpels. Finally, the two fourth-whorl carpels are fused to form a normal appearing gynoecium, although abnormalities in carpel fusion are frequently observed in the late arising or distal flowers along the primary inflorescence (data not shown).

ap2-10 does not visibly alter vegetative growth under standard long-day growth conditions (data not shown). However, we found that *ap2-10* seed are structurally defective by comparing wild-type and mutant seed using scanning electron microscopy. As shown in Figures 1E and 1F, the epidermal cells of the testa or seed coat of wild-type seed are distinguished by a single prominent "epidermal plateau" or columella that is formed during seed development and is associated with the storage of complex polysaccharides or seed mucilage (Koornneef, 1981; Goto, 1982; Léon-Kloosterziel et al., 1994; B.G.W. den Boer, K.D. Jofuku, and J.K. Okamuro, unpublished results). By contrast, Figures 1G and 1H show that the epidermal cells of *ap2-10* seed clearly lack this structure and are larger and less regular in shape compared to wild-type cells. One consequence of this defect is that mutant seeds are rendered hypersensitive to NaOCl treatment (data not shown). We analyzed seed from eight independent *ap2* mutants (*ap2-2* through *ap2-9*) and confirmed that they all display this defect in seed coat development and are NaOCl hypersensitive (data not shown). Although we do not know precisely when during seed development these epidermal cells differentiate, the mutant seed phenotype indicates that *AP2* gene expression and gene function are not restricted to flower ontogeny. Together, these data demonstrate that *AP2* is required for the normal development of both floral and nonfloral structures.

AP2 Gene Isolation

We determined that *ap2-10* was the product of a T-DNA insertion mutation by genetic linkage analysis using the T-DNA–encoded neomycin phosphotransferase II (NPTII) gene as a genetic marker (Methods). We selected an overlapping set of T-DNA–containing recombinant phage from an *ap2-10* genome library and used the plant DNA sequences flanking the T-DNA insertion element as hybridization probes to isolate phage containing the corresponding region from a wild-type Arabidopsis genome library (see Methods). Figure 2A schematically shows the 30.2-kb wild-type *AP2* gene region. We mapped the site of T-DNA insertion in *ap2-10* to a 7.2-kb EcoRI fragment centrally located within the *AP2* gene region (Figure 2A). We introduced the wild-type 7.2-kb fragment into *ap2-1* plants to test for complementation of the mutant flower and seed coat phenotypes shown in Figures 2B to 2D. Figure 2E shows a typical flower from one of 17 independent transgenic *ap2-1* plants containing the wild-type 7.2-kb genomic fragment. All flowers produced by these plants were similar in phenotype and produced a wild-type complement of four sepals, four petals, six stamens, and a normal gynoecium. Moreover, the seeds produced by these flowers had wild-type seed coats, as shown in Figures 2F and 2G. In a second series of experiments, *ap2-10* flower and seed coat phenotypes were also complemented by the introduction of the 7.2-kb DNA fragment (B.G.W. den Boer and M. Van Montagu, data not shown). Together, these experiments demonstrate that we have isolated the *AP2* gene.

This content downloaded from 164.67.109.135 on Thu, 17 Jul 2014 21:23:12 PM
All use subject to JSTOR Terms and Conditions

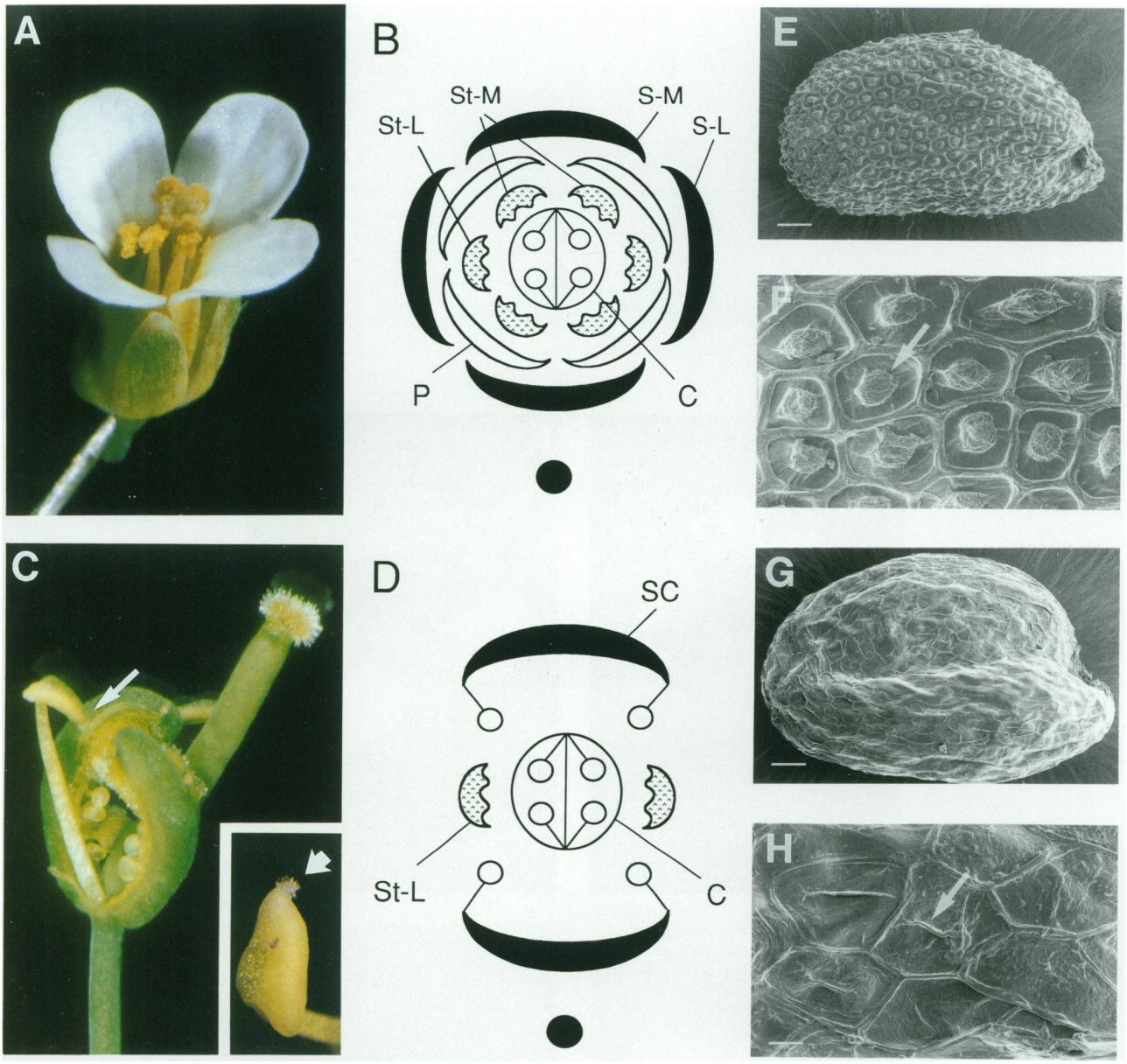

Figure 1. Morphological Characterization of *ap2-10* Mutant Flowers.

(A) Wild-type Arabidopsis flower.

(B) Diagram of a wild-type Arabidopsis flower. The organs of the mature flower are organized into four concentric rings or whorls. The first and outermost whorl contains four sepals—two medial organs (S-M) and two lateral organs (S-L). The second whorl contains four petals (P) that are alternating in position with sepals. The third whorl consists of six stamens—four long medial organs (St-M) and two short lateral organs (St-L). The fourth whorl consists of a gynoecium composed of two fused ovule-bearing carpels (C). The filled circle indicates the position of the inflorescence stem.

(C) *ap2-10* (T10) mutant flower.

(D) Diagram of an *ap2-10* mutant flower. First-whorl organs range in number from two to four. Medial organs are mosaic ovule-bearing sepal–carpels (SC) displaying abundant stigmatic papillae along the organ margins. Lateral first-whorl organs, if present, are leaflike. Second-whorl petals and third-whorl medial stamens are absent in *ap2-10* flowers. Lateral stamens (St-L) are characterized by stigmatic papillae on the distal tips of each anther as indicated by an arrow and shown in the inset in **(C)**. Two fourth-whorl carpels form a normal appearing gynoecium. The filled circle indicates the position of the inflorescence stem.

(E) Scanning electron micrograph of a wild-type Arabidopsis seed. Bar = 50 μm.

(F) Magnified image of the wild-type seed coat from **(E)** showing testa epidermal cell morphology. The arrow indicates the raised central structure referred to as the epidermal plateau or columella that is present in each testa cell. Bar = 5 μm.

(G) Scanning electron micrograph of an *ap2-10* mutant seed. Bar = 50 μm.

(H) Magnified image of the mutant seed coat from **(G)**. The arrow indicates the absence of the central epidermal plateau characteristic of wild-type cells shown in **(E)** and **(F)**. Bar = 5 μm.

This content downloaded from 164.67.109.135 on Thu, 17 Jul 2014 21:23:12 PM
All use subject to JSTOR Terms and Conditions

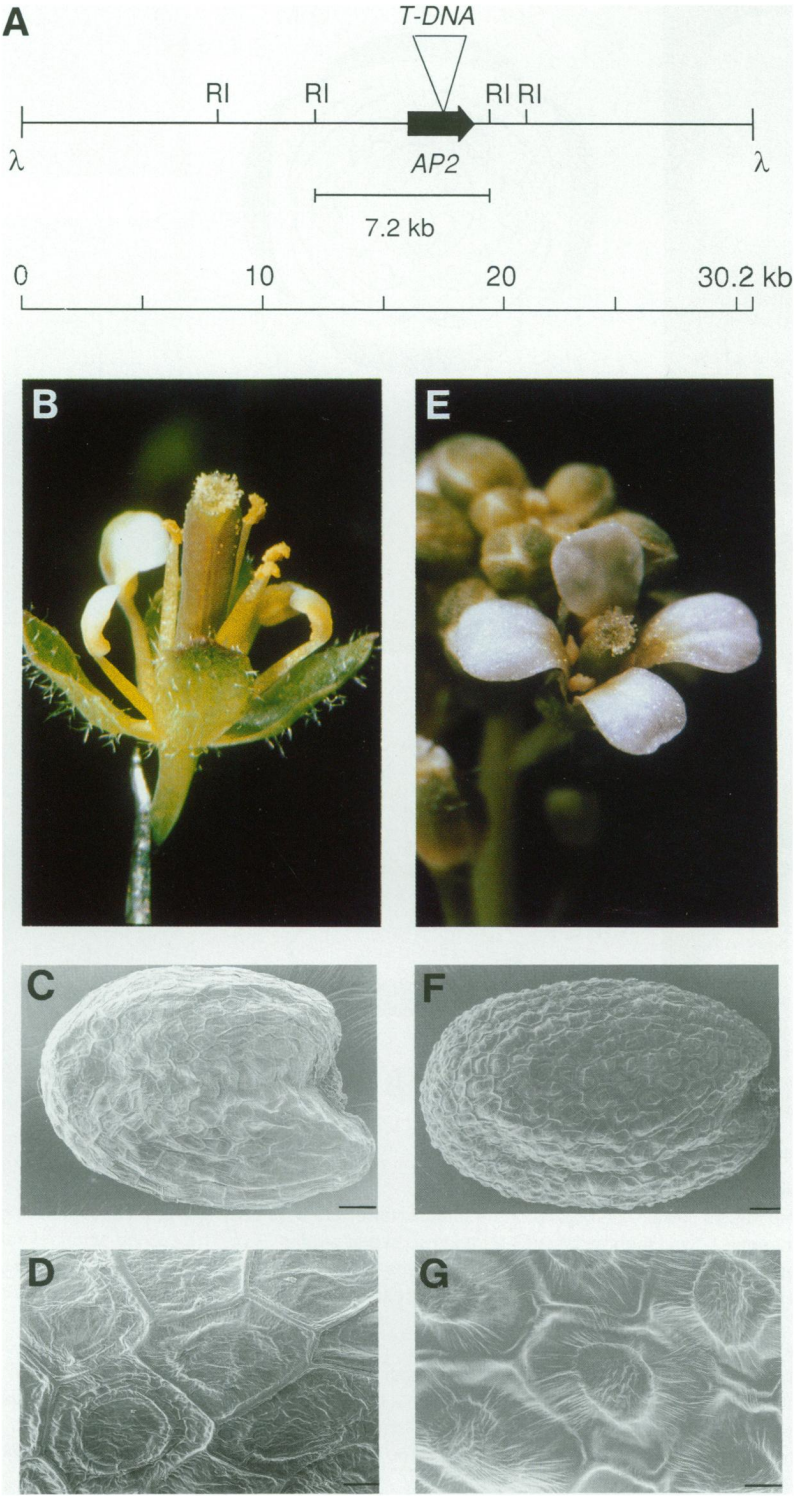

Figure 2. Molecular Complementation of the *ap2* Mutation.

This content downloaded from 164.67.109.135 on Thu, 17 Jul 2014 21:23:12 PM
All use subject to JSTOR Terms and Conditions

AP2 Encodes a Putative Nuclear Factor

We isolated and characterized five Arabidopsis flower cDNA clones corresponding to sequences within the 7.2-kb *AP2* gene region to begin to characterize the *AP2* gene. We confirmed that all five cloned cDNAs represented *AP2* gene transcripts by using an antisense gene strategy to induce *ap2* mutant flowers in wild-type plants (P. Wesley and J.K. Okamuro, unpublished results). To determine *AP2* gene structure, we compared the nucleotide sequences of the cDNA inserts to that of the 7.2-kb *AP2* genomic fragment. Figure 3 shows that the *AP2* gene is 2.5 kb in length and contains 10 exons and nine introns that range from 85 to 110 bp in length.

Figure 4 shows that the *AP2* gene encodes a theoretical polypeptide of 432 amino acids with a predicted molecular mass of 48 kD. We compared the *AP2* nucleotide and predicted protein sequences with a merged, nonredundant data base (Methods) and found that *AP2* has no significant global similarity to any known regulatory protein. Our sequence analysis, however, did reveal the presence of several sequence features that may be important for AP2 protein structure or function. First, AP2 contains a 37–amino acid serine-rich acidic domain (amino acids 14 to 50) (Figure 4) that is analogous to regions that function as activation domains in a number of RNA polymerase II transcription factors (Mitchell and Tjian, 1989). Second, AP2 has a highly basic 10–amino acid domain (amino acids 119 to 128) that includes a putative nuclear localization sequence KKSR (Dingwall and Laskey, 1986; Chelsky et al., 1989; Garcia-Bustos et al., 1991; Howard et al., 1992; Varagona et al., 1992), suggesting that AP2 may function in the nucleus. Finally, Figures 4 and 5 show that the central core of the AP2 polypeptide (amino acids 129 to 288) contains two copies of a 68–amino acid direct repeat that we refer to as the AP2 domain. Figure 5A shows that the two copies of this repeat, designated AP2-R1 and AP2-R2, share 53% amino acid identity and 69% amino acid homology. Each AP2 repeat contains an 18–amino acid conserved core region that shares 83% amino acid homology. Figure 5B shows that both copies of this core region are theoretically capable of forming amphipathic α-helical structures that may participate in protein–protein interactions.

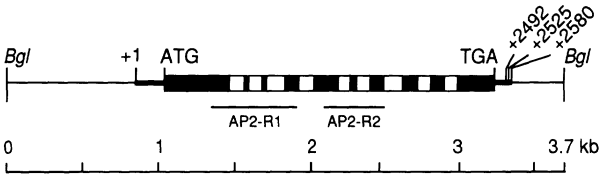

Figure 3. *AP2* Gene Structure.

The *AP2* gene is contained within the 3.7-kb BglII (Bgl) DNA fragment. The *AP2* transcription start site and the three alternative polyadenylation sites were determined by primer extension (P. Wesley and J.K. Okamuro, unpublished results) and cDNA sequence analysis (see Methods); they are designated by +1 and +2492, +2525, and +2580, respectively. The thickened lines represent the 5′ and 3′ untranslated gene regions. ATG and TGA indicate the positions of the putative translational start and stop codons, respectively. Solid and open boxes represent exons and introns, respectively. AP2-R1 and AP2-R2 indicate regions encoding the AP2 domain repeated motif shown in Figures 4 and 5.

DNA sequence analysis of three *ap2* mutant alleles suggests that the AP2 domain is essential for AP2 function. As shown in Figure 5A, the temperature-sensitive mutations in *ap2-1* and *ap2-5* (Koornneef et al., 1980; Bowman et al., 1989; Kunst et al., 1989) disturb similar regions in each copy of the AP2 domain (residues Gly-251 in *ap2-1* and Gly-159 in *ap2-5*). *ap2-5* also differs from wild-type AP2 at position Gln-420 (Figure 4). By contrast, the phenotypically strong *ap2-10* mutant gene is interrupted by a T-DNA insertion in exon 7 that encodes the C-terminal half of AP2-R2 (Figures 4 and 5). Taken together, these findings suggest that *AP2* encodes a putative nuclear protein and that the AP2 domain is critical to the functioning of this protein in the cell.

AP2 Gene Transcripts Are Not Flower Specific

We hybridized labeled *AP2* DNA to an RNA gel blot containing flower and vegetative leaf polysomal poly(A) mRNAs to determine whether *AP2* gene expression was spatially and temporally restricted to flower development. Figure 6A shows that

Figure 2. Molecular Complementation of the *ap2* Mutation.

(A) Schematic diagram of the *AP2* gene region. Three overlapping Arabidopsis Ler genomic clones were used to assemble a 30.2-kb physical map of the region flanking the *ap2-10* T-DNA insertion site (see Methods). EcoRI (RI) restriction sites are shown for reference. The arrow represents the *AP2* gene. The 7.2-kb EcoRI fragment that was used in molecular complementation experiments is indicated.
(B) *ap2-1* mutant flower. First-whorl organs are leaves, distinguished by the presence of stellate trichomes. Second-whorl organs are stamenoid petals. Third- and fourth-whorl organs appear morphologically wild type.
(C) Scanning electron micrograph of an *ap2-1* mutant seed. Bar = 50 μm.
(D) Magnified image of the mutant seed coat shown in **(C)**. The central epidermal plateau is dramatically reduced compared to that in the wild-type seed (Figures 1E and 1F). Bar = 5 μm.
(E) Transgenic *ap2-1* flower containing the 7.2-kb wild-type *AP2* gene region. All four organ types are morphologically wild type.
(F) Scanning electron micrograph of a transgenic *ap2-1* seed containing the wild-type *AP2* gene. Bar = 100 μm.
(G) Magnified image of the transgenic *ap2-1* seed shown in **(E)**. Testa epidermal cells are indistinguishable from the wild type. Bar = 5 μm.

This content downloaded from 164.67.109.135 on Thu, 17 Jul 2014 21:23:12 PM
All use subject to JSTOR Terms and Conditions

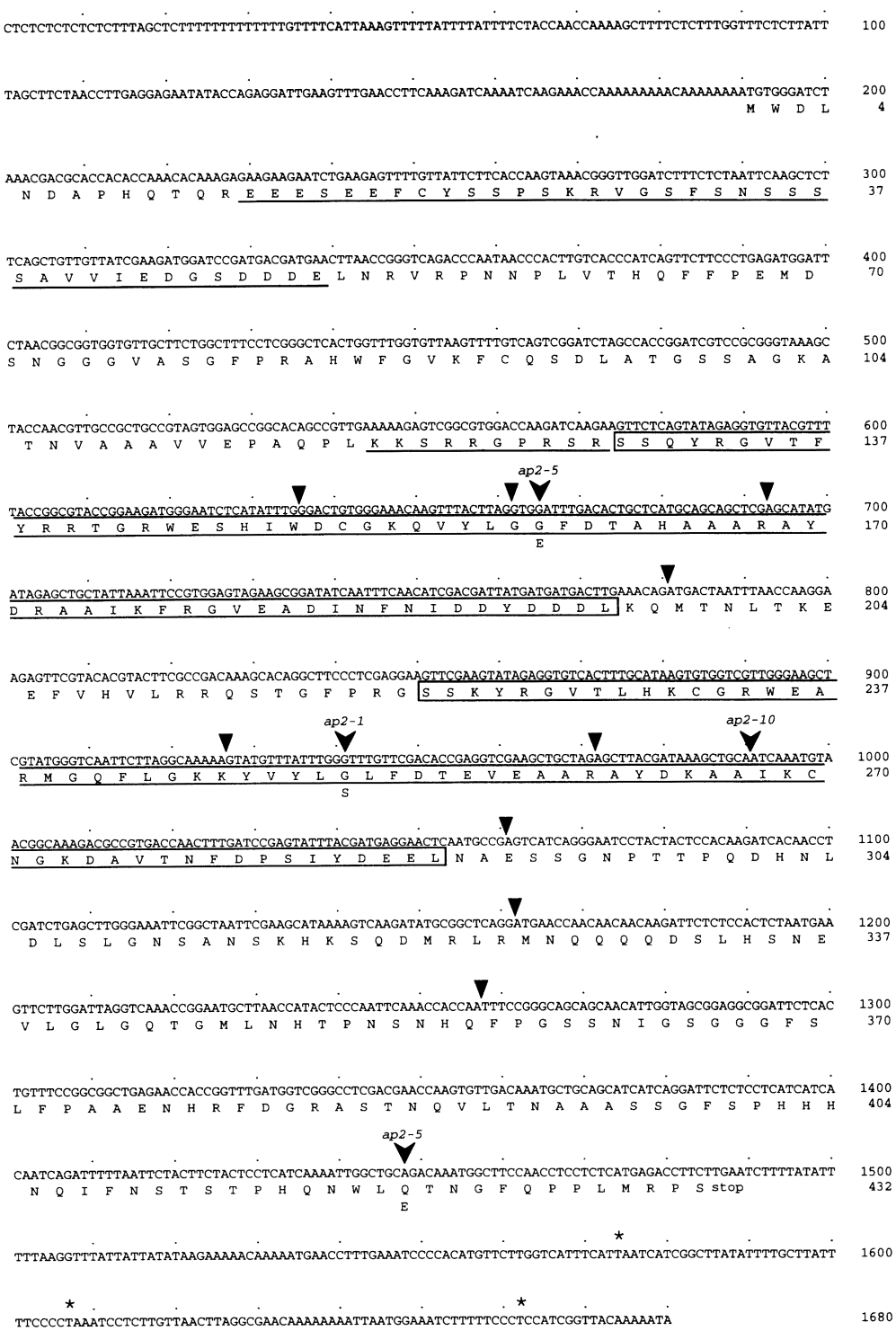

Figure 4. *AP2* Gene Sequence.

The *AP2* nucleotide sequence shown was derived from flower cDNA clones λAP2c1, λAP2c3, and λAP2c5 (Methods). The corresponding amino acid sequence is shown below the nucleotide sequence. Numbers to the right of the sequences refer to the positions of nucleotide and amino acid residues. Inverted triangles mark intron–exon boundaries. Arrowheads indicate the positions of the *ap2-1*, *ap2-5*, and *ap2-10* mutations. The amino acid substitutions in *ap2-1* and *ap2-5* are shown below the AP2 amino acid sequence. Asterisks designate the positions of the three alternative poly(A) addition sites. Boxes delineate the AP2 domain repeated motif AP2-R1 (amino acids 129 to 195) and AP2-R2 (amino acids 221 to 288). A highly acidic, serine-rich region (amino acids 14 to 50) that may serve as a transcriptional activation domain and a 10–amino acid basic region (amino acids 119 to 128) that contains a putative nuclear localization signal sequence (KKSR) are underlined. The GenBank accession number for the *AP2* sequence is U12546.

This content downloaded from 164.67.109.135 on Thu, 17 Jul 2014 21:23:12 PM
All use subject to JSTOR Terms and Conditions

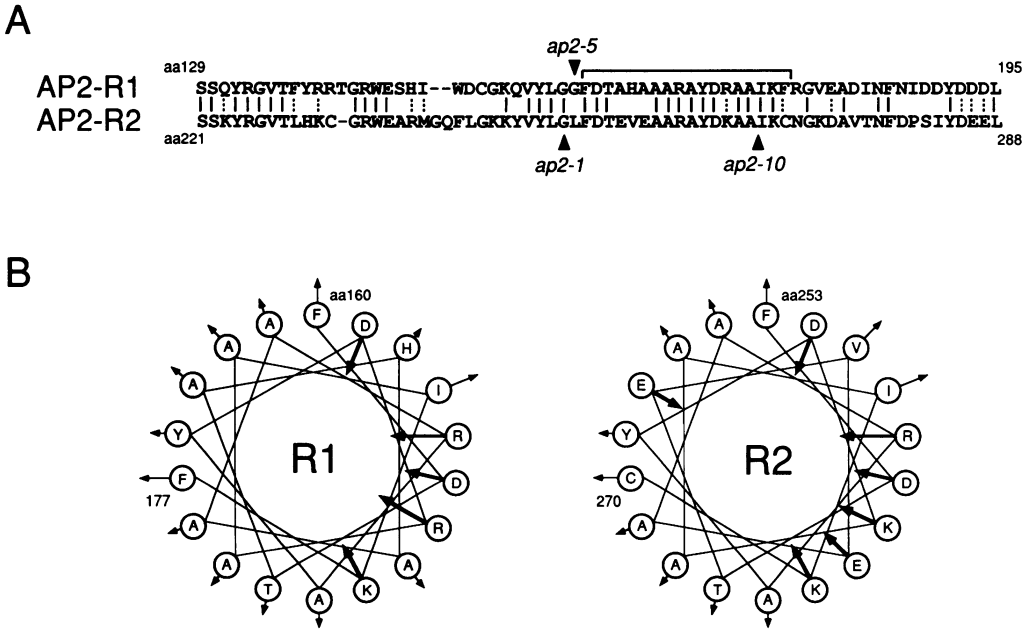

Figure 5. AP2 Domain.

(A) Amino acid (aa) sequence alignment between AP2 direct repeats AP2-R1 (amino acids 129 to 195) and AP2-R2 (amino acids 221 to 288). Solid and dashed lines between the two sequences indicate residue identity and similarity, respectively. Arrowheads indicate the positions of the *ap2-1*, *ap2-5*, and *ap2-10* mutations described in Figure 4. The bracket above the AP2-R1 and AP2-R2 sequences indicates the residues capable of forming amphipathic α-helices shown in **(B)**.

(B) Schematic diagrams of the putative AP2-R1 (R1) and AP2-R2 (R2) amphipathic α-helices. The N-terminal ends of the R1 and R2 helices begin at amino acid residues (aa) Phe-160 and Phe-253 and rotate clockwise by 100° per residue through Phe-177 and Cys-270, respectively. Arrows directed toward or away from the center of the helical wheel diagrams indicate the negative or positive degree of hydrophobicity as defined by Jones et al. (1992).

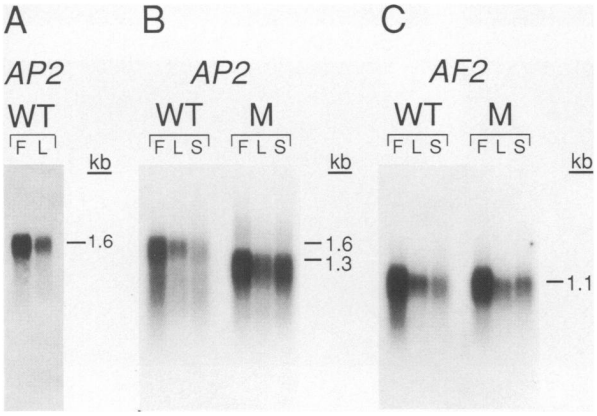

Figure 6. *AP2* Gene Expression in Arabidopsis.

Wild-type and *ap2-10* mutant flower (F), leaf (L), and stem (S) polysomal poly(A) mRNAs were fractionated on denaturing agarose gels, transferred to nylon membranes, and reacted with labeled plasmid DNA probes as outlined in Methods. Flower mRNAs were isolated from inflorescences containing buds at floral stages 1 to 10 (Smyth et al., 1990). Leaf and stem mRNAs were isolated from young unexpanded rosette leaves and inflorescence stem internode tissue, respectively.

(A) *AP2* gene expression in wild-type (WT) flower and leaf. Labeled *AP2* cDNA plasmid DNA was reacted with an mRNA gel blot containing flower and leaf mRNA. Lanes F and L each contain 5 μg of mRNA.

(B) *AP2* gene expression in wild-type and *ap2-10* mutant plants. Labeled *AP2* cDNA plasmid DNA was hybridized to a gel blot containing mRNA from wild-type (WT) and mutant (M) plants. Lanes F, L, and S contain 5, 3, and 4 μg of mRNA, respectively.

(C) *AF2* gene expression in wild-type and *ap2-10* mutant plants. The mRNA gel blot from **(B)** was stripped of probe and rehybridized with labeled *AF2* cDNA plasmid DNA.

a 1.6-kb *AP2* mRNA was detected at low levels in both flowers (lane F) and leaves (lane L). We estimated that the prevalence of the *AP2* flower transcripts was ~0.002% of the flower mRNA population (Methods). By contrast, the *AP2* mRNA prevalence in leaves was ~10-fold lower.

We hybridized *AP2* DNA to an RNA gel blot containing wild-type and *ap2-10* flower, leaf, and stem mRNAs to determine whether *AP2*, or a gene related to *AP2*, was active in nonfloral organs. We assumed that the presence of T-DNA vector sequences in the *ap2-10* gene would result in the absence of *ap2-10* gene transcripts or the production of an altered transcript that could be distinguished from unaltered transcripts produced by *AP2*-related genes in the Arabidopsis genome.

This content downloaded from 164.67.109.135 on Thu, 17 Jul 2014 21:23:12 PM
All use subject to JSTOR Terms and Conditions

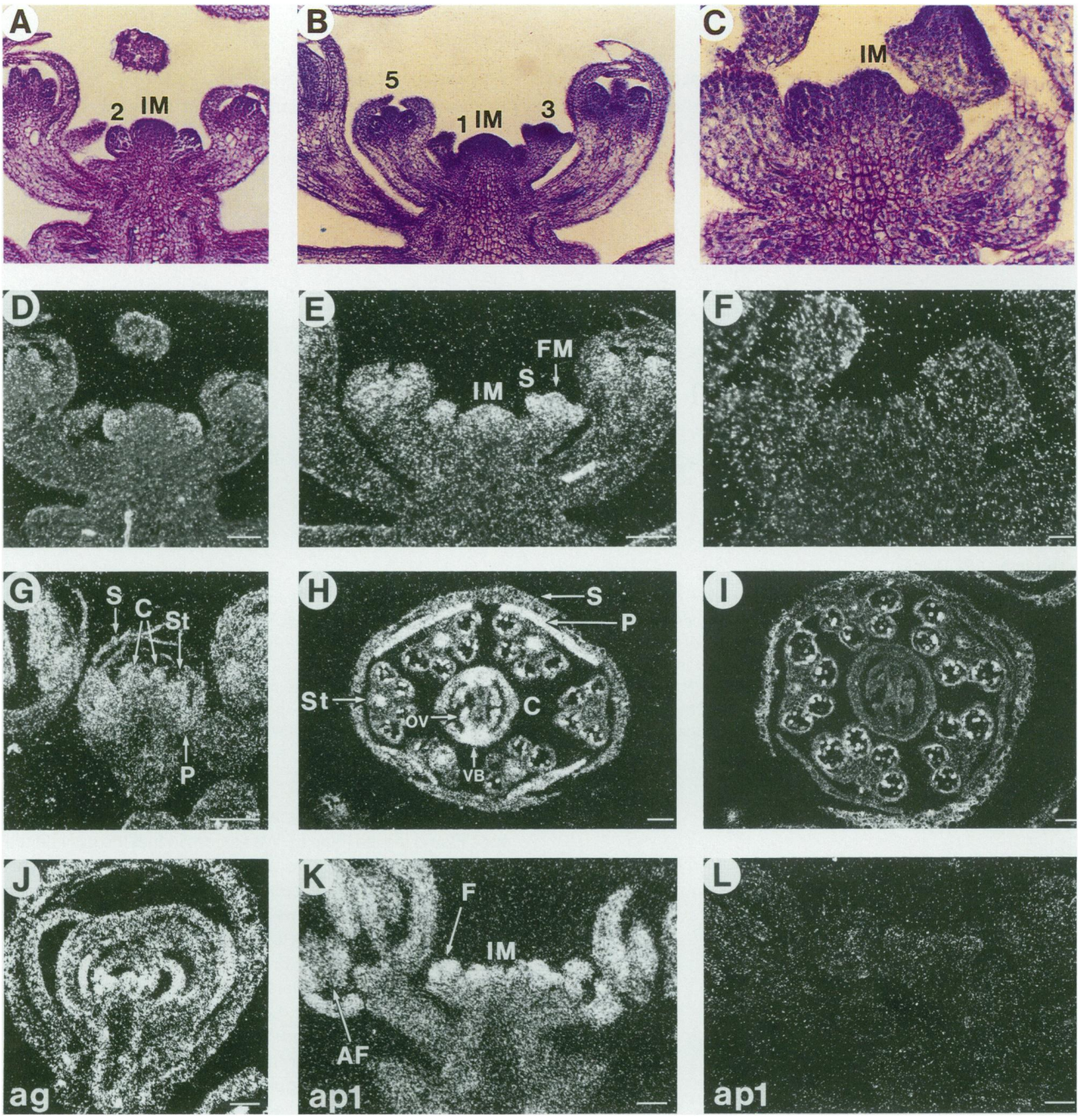

Figure 7. Cellular Localization of *AP2* mRNA in Wild-Type and Mutant Arabidopsis Flowers.

Wild-type flowers at stages 1 through 12 of development as defined by Smyth et al. (1990) were fixed, embedded in paraffin, sliced into 10-μm sections, and hybridized with single-stranded ^{35}S-labeled RNA probes as outlined in Methods. Photographs were taken using bright-field and dark-field microscopy. Film emulsion exposure times for **(D)**, **(E)** to **(I)**, and **(J)** to **(L)** were performed at 4°C for 2.5, 6, and 10 weeks, respectively. Numbers indicate floral developmental stages. IM and FM designate the inflorescence and floral meristems, respectively. Bars in **(D)** to **(L)** = 25 μm.

(A) to **(C)** Bright-field micrographs of longitudinal sections of wild-type inflorescences containing the inflorescence meristem and flowers at stages 1 to 5 of development. Sections were stained with toluidine blue.

(D) and **(E)** In situ hybridization of an *AP2* anti-mRNA probe to longitudinal sections of wild-type inflorescences containing the inflorescence meristem and flowers at stages 1 to 5 of development. White grains represent regions containing *AP2* RNA–RNA hybrids. S refers to sepal primordia.

(F) In situ hybridization of an *AP2* mRNA probe to longitudinal sections of wild-type inflorescences containing the inflorescence meristem and flowers at stages 1 to 4 of development. White grains represent background hybridization levels.

This content downloaded from 164.67.109.135 on Thu, 17 Jul 2014 21:23:12 PM
All use subject to JSTOR Terms and Conditions

Figure 6B shows that the 1.6-kb wild-type *AP2* transcript was not detectable in *ap2-10* mutant flower, leaf, or stem mRNAs (compare lanes under WT and M). Instead, a 1.3-kb transcript was detected in all three mutant mRNAs at prevalence levels higher than observed in wild-type plants.

We tested whether the truncated transcripts reflected a true shift in *ap2* mRNA size in *ap2-10* by rehybridizing the gel blot shown in Figure 6B to labeled *AF2* cDNA. *AF2* represents a gene that maps ~7 kb upstream from *AP2*, is expressed in floral and vegetative tissues, and encodes a 1.1-kb mRNA. DNA sequence analysis suggests that *AF2* encodes an inosine-5′-monophosphate dehydrogenase (data not shown). Figure 6C shows that the 1.1-kb *AF2* mRNA transcript was detected in both wild-type and mutant mRNAs at similar prevalence levels. We conclude from these data that the *AP2* gene is expressed at the mRNA level in flowers, leaves, and stems and that *AP2*-related gene transcripts are not detectable in these organs under moderately stringent hybridization conditions.

AP2 Is Expressed in the Inflorescence Meristem and in All Four Types of Floral Organs

We reacted *AP2* probes with floral sections in situ (Methods) to determine when and where during flower ontogeny *AP2* gene expression occurs. The bright-field micrographs in Figures 7A to 7C show longitudinal sections of the inflorescence meristem and wild-type floral buds at several stages of development (Smyth et al., 1990). The dark-field micrographs in Figures 7D and 7E show that *AP2* transcripts are present at low levels throughout the apical region of the shoot. In addition, there is a higher concentration of grains in the inflorescence meristem (Figure 7E) and in stages 1 and 2 floral buds (Figures 7D and 7E). By contrast, Figure 7F shows that only background hybridization grains were observed in a control experiment.

Figure 7E shows that in stage 3 flowers, the domain of *AP2* gene transcripts spreads from the floral meristem to include the sepal primordia. By stages 6 through 7 of flower development, the domain of *AP2* gene transcripts has expanded to include the petal, stamen, and carpel primordia (Figure 7G). Finally, late in flower development, *AP2* transcripts were still detectable in all organs but were most highly concentrated in petals and in specific regions or cell types within each reproductive floral organ system (compare Figures 7H and 7I). For example, high concentrations of grains were observed in the vascular tissue of stamens, the medial vascular bundles of carpels, and in developing ovules. Together, these results indicate that *AP2* transcripts are detectable at low levels throughout the shoot apex and at enhanced levels in the inflorescence meristem, in young floral buds, and throughout the early stages of Arabidopsis flower development and organogenesis. Finally, late during floral organ differentiation, *AP2* transcripts become spatially restricted to specific organ, tissue, and cell types within the flower.

We analyzed *AP2* gene expression in *ag-1* and *ap1-1* mutant flowers to determine whether *AG* or *AP1* were required for establishing the wild-type pattern of *AP2* gene expression during flower development. AG and AP1 belong to a family of flower-specific transcription factors that regulate both floral meristem and floral organ identity in Arabidopsis (Yanofsky et al., 1990; Mandel et al., 1992b). Figure 7J shows that in *ag-1* mutant flowers, *AP2* transcripts are detectable throughout the mutant flower and in all floral organs. Figure 7K shows that in *ap1-1* mutant flowers, *AP2* transcripts are detectable throughout the mutant inflorescence, in each mutant flower, and in all axillary floral buds. By contrast, no hybridization grains were observed above background levels in a control experiment (Figure 7L). We conclude from these results that neither *AP1* nor *AG* is required for *AP2* gene activity during flower development.

DISCUSSION

AP2 is a central player in the gene network controlling Arabidopsis flower homeotic gene expression and flower development. In this study, we showed that *AP2* is also required during seed development. Our analysis of *AP2* gene expression at the RNA level revealed that, unlike other floral homeotic genes, *AP2* is expressed in both nonfloral and floral tissues and organs.

(G) In situ hybridization of an *AP2* anti-mRNA probe to a longitudinal section of a wild-type stage 7 flower. S, P, St, and C indicate sepal, petal, stamen, and carpel primordia, respectively.

(H) In situ hybridization of an *AP2* anti-mRNA probe to a transverse section of a wild-type stage 12 flower. S, sepal; P, petal; St, stamen; C, carpel; OV, ovule; VB, vascular bundle.

(I) In situ hybridization of an *AP2* mRNA probe to a transverse section of a wild-type stage 12 flower. Background fluorescence is observed in pollen grains and tapetum.

(J) In situ hybridization of an *AP2* anti-mRNA probe to a longitudinal section of an *ag* mutant flower. *ag-1* mutant flowers are characterized by the absence of carpels and the homeotic conversion of stamens to petals. In addition, the floral meristem displays an indeterminate or repetitive pattern of organogenesis that results in a structure [(sepal, petal, petal)$_n$ where $n > 1$] referred to as a flower-within-a-flower (Bowman et al., 1989).

(K) In situ hybridization of an *AP2* anti-mRNA probe to a longitudinal section of an *ap1* mutant inflorescence. *ap1-1* mutant flowers are characterized by the homeotic conversion of sepals into bractlike leaves, the absence of petals, and a normal complement of stamens and carpels (Irish and Sussex, 1990; Bowman, 1992; Bowman et al., 1993; Schultz and Haughn, 1993). *ap1-1* flowers are also characterized by the ectopic production of secondary flowers in the axils of the first-whorl floral organs, indicating that the floral meristem is partially converted into an inflorescence. F, flower; AF, axillary flower.

(L) In situ hybridization of an *AP2* mRNA probe to a longitudinal section of an *ap1* mutant inflorescence.

This content downloaded from 164.67.109.135 on Thu, 17 Jul 2014 21:23:12 PM
All use subject to JSTOR Terms and Conditions

We therefore propose that *AP2* has a more expanded role in Arabidopsis development than previously recognized.

AP2 Represents a New Class of Plant Regulatory Protein

Our studies revealed that *AP2* encodes a theoretical polypeptide of 432 amino acids that is distinguished by a novel 68–amino acid repeated motif, the AP2 domain (Figures 4 and 5). This motif is conserved between highly divergent *AP2*-like gene family members in Arabidopsis (B.G.W. den Boer, K.D. Jofuku, and J.K. Okamuro, unpublished results) and is also present in *AP2*-like genes isolated from petunia, snapdragon, and rice (B.G.W. den Boer, A. Gerats, M. Van Montagu, E. Coen, and J.K. Okamuro, unpublished results). One important conclusion from our characterization of the mutant genes *ap2-1* and *ap2-5* is that the AP2 domain is necessary for *AP2* genetic functions, including the establishment of floral meristem identity (Irish and Sussex, 1990; Bowman, 1992; Huala and Sussex, 1992; Bowman et al., 1993; Schultz and Haughn, 1993; Shannon and Meeks-Wagner, 1993), the specification of sepal and petal organ identity (Haughn and Somerville, 1988; Bowman et al., 1989, 1991b; Kunst et al., 1989), and the spatial control of *AG* homeotic gene expression (Bowman et al., 1991a; Drews et al., 1991a).

ap2-1 and *ap2-5* are phenotypically weak mutants that can display a continuous spectrum of related flower phenotypes on a single plant (Bowman et al., 1989; Kunst et al., 1989; B.G.W. den Boer, K.D. Jofuku, and J.K. Okamuro, unpublished observations). Both *ap2-1* and *ap2-5* contain substitutions at glycine residues located at similar positions within the two AP2 domain repeats (Figures 4 and 5). One consequence of these changes may be the disruption of an element of AP2 structure that is essential for its function. The AP2 domain contains an 18–amino acid conserved core region that is theoretically capable of forming an amphipathic α-helix (Figure 5). The replacement of glycine residues with either Ser (*ap2-1*) or Glu (*ap2-5*) may perturb the formation or the position of these α-helical structures within the AP2 protein structure. Amphipathic α-helices are capable of mediating protein–protein interactions through the formation of coiled-coil structures (reviewed by Segrest et al., 1990) and may therefore function similarly in AP2. Thus, one function of this region may be to mediate AP2 dimer formation through the interaction of these amphipathic helices as in the Epstein-Barr virus transcription factor ZEBRA (Flemington and Speck, 1990). Alternatively, it may facilitate AP2 interactions with its genetic partners LFY or AP1.

AP2 promotes the establishment of the floral meristem in cooperation with at least three other control genes, *AP1*, *CAL*, and *LFY* (Irish and Sussex, 1990; Bowman, 1992; Huala and Sussex, 1992; Bowman et al., 1993; Schultz and Haughn, 1993; Shannon and Meeks-Wagner, 1993). Although the molecular basis of these genetic interactions is not yet known, we hypothesize that the AP2 protein may function as a nuclear transcription factor because of its interactions with *AP1* and *LFY*, which are presumed to function in the nucleus (Mandel et al., 1992b; Weigel et al., 1992), and because of its role in the regulation of *AG* gene expression (Bowman et al., 1991a; Drews et al., 1991a). This hypothesis is supported by the fact that AP2 contains a 10–amino acid highly basic domain that includes a putative nuclear localization signal sequence KKSR (Chelsky et al., 1989) as well as a 37–amino acid acidic and serine-rich domain (Figure 4) that may function as an activation domain. Acidic activation domains represent one of three classes of transcription factor sequences that can stimulate basal levels of gene transcription (Mitchell and Tjian, 1989). Several serine residues in the AP2 acidic domain could serve as sites of protein phosphorylation and thus provide a molecular mechanism for controlling AP2 activity during Arabidopsis development (Hunter and Karin, 1992).

Spatial Distribution of *AP2* Gene Transcripts Is Not Determined by the Homeotic Gene *AG*

The results of our molecular and genetic analysis of *AP2* gene expression during Arabidopsis flower development are consistent with the pleiotropic effects of *ap2* mutations on floral meristem identity (Irish and Sussex, 1990; Huala and Sussex, 1992; Bowman et al., 1993; Schultz and Haughn, 1993; Shannon and Meeks-Wagner, 1993) and floral organ identity and development (Figure 1) (Komaki et al., 1988; Bowman et al., 1989, 1991a; Kunst et al., 1989). Our in situ hybridization analysis of *AP2* gene expression in wild-type plants showed that *AP2* transcripts are detectable throughout the floral meristem and that the concentration of *AP2* transcripts increased dramatically in young emerging flower primordia (Figure 7). We showed that the domain of *AP2* gene expression expanded continuously and uniformly during early flower development from the floral primordium to all four types of floral organ primordia—sepal, petal, stamen, and carpel (Figure 7). The presence of *AP2* transcripts in both stamens and carpels is consistent with the observation that strong *ap2* mutants are defective in stamen number and development as well as in carpel fusion and ovule development (Figure 1) (Komaki et al., 1988; Kunst et al., 1989; Bowman et al., 1991b; Modrusan et al., 1994). Thus, *AP2* is clearly expressed in and functions during the development of all four types of floral organs.

The sustained expression of *AP2* during Arabidopsis inflorescence and early flower development provides a striking contrast to the sequential and spatially overlapping expression patterns of the MADS-box family of floral homeotic regulatory genes *AG*, *AP1*, and *AP3* (Yanofsky et al., 1990; Bowman et al., 1991a; Drews et al., 1991a; Jack et al., 1992; Mandel et al., 1992b). Although the genetic model for the control of floral organ identity (Haughn and Somerville, 1988; Coen and Meyerowitz, 1991; Meyerowitz et al., 1991; Bowman et al., 1993; Gustafson-Brown et al., 1994) has accurately predicted the spatial expression patterns for *AG*, *AP3*, and *AP1* (Yanofsky et al., 1990; Drews et al., 1991a; Jack et al., 1992; Mandel et al., 1992b; Gustafson-

This content downloaded from 164.67.109.135 on Thu, 17 Jul 2014 21:23:12 PM
All use subject to JSTOR Terms and Conditions

Brown et al., 1994), our data show that this is not the case for *AP2*. *AP2* expression is not restricted to sepals and petals as was predicted by this model. Furthermore, if *AG* controls flower development through its activity as a transcription factor (Yanofsky et al., 1990; Ma et al., 1991; Shiraishi et al., 1993), then our in situ hybridization experiments clearly demonstrate that *AP2* and *AG* gene activities are not mutually antagonistic. That is, *AG* does not repress the appearance of *AP2* gene transcripts in stamens and carpels, nor does *AP2* repress *AG* expression in these organs as it does in sepals and petals (Bowman et al., 1991a; Drews et al., 1991a).

Our conclusion that *AG* does not negatively regulate *AP2* gene expression during flower development is also supported by the observation that *ap2* gene transcripts accumulate in *ap2-10* flowers (Figure 6). *ap2-10* is phenotypically a strong mutant showing the transformation of sepals to carpels and the suppression of second-whorl organ formation and development (Figure 1) that we presume results from the ectopic expression of *AG* in first- and second-whorl organ primordia (Bowman et al., 1991a; Drews et al., 1991a; Mandel et al., 1992a; Mizukami and Ma, 1992). The genetic model for the control of Arabidopsis floral organ identity predicts that in strong *ap2* mutants, mutant gene transcription should be suppressed compared to wild-type flowers as a consequence of ectopic *AG* gene expression (Coen and Meyerowitz, 1991; Drews et al., 1991b; Meyerowitz et al., 1991). We showed by RNA gel blot analysis, however, that the *ap2-10* gene is still transcriptionally active in mutant flowers and that the prevalence of *ap2-10* RNA in mutant flowers is equal to, if not greater than, the steady state level of *AP2* transcripts in wild-type flowers (Figure 6). Thus, the analysis of *AP2* gene expression by in situ hybridization and RNA gel blot experiments strongly suggest that *AG* does not determine the spatial pattern of *AP2* gene activity.

AP2 and *AG* are expressed together in developing stamens and carpels; yet in sepals and petals, *AP2* negatively regulates *AG* gene expression (Drews et al., 1991a). This genetic and molecular paradox raises the question—how does *AP2* suppress *AG* gene transcription in sepals and petals but not in stamens and carpels? One hypothesis is that *AP2* works cooperatively with another regulatory gene or genes to suppress *AG* gene expression in sepals and petals (Bowman et al., 1993; Schultz and Haughn, 1993). The activity of this genetic partner should be restricted to first- and second-whorl organs. One possible partner is the homeotic gene *AP1* (Irish and Sussex, 1990; Mandel et al., 1992a; Gustafson-Brown et al., 1994). *AP1* and *AP2* act synergistically to establish and control floral meristem identity (Irish and Sussex, 1990; Huala and Sussex, 1992; Bowman et al., 1993; Schultz and Haughn, 1993; Shannon and Meeks-Wagner, 1993). Moreover, *AP1* gene expression in wild-type flowers is restricted to sepals and petals (Mandel et al., 1992a). A recent study has shown that *AG* is ectopically expressed in flowers, stems, and leaves in *ap1 lfy* double mutants (Weigel and Meyerowitz, 1993). By contrast, the spatial pattern of *AG* gene expression is not altered in *ap1* mutant flowers (Mandel et al., 1992a). Therefore, we conclude that the pattern of *AG* gene expression during flower development is not negatively controlled by *AP2* alone, as predicted, but perhaps by the cooperative actions of *AP2*, *AP1*, and *LFY*.

AP2 Continues to Function after Floral Ontogeny

Unlike other floral homeotic genes, *AP2* is also required for normal seed coat development. We showed that mutations in *ap2* result in the absence of a prominent seed epidermal cell structure referred to as the epidermal plateau or columella (Figures 1 and 2). A similar observation was recently reported for *ap2-1* mutant seed (Léon-Kloosterziel et al., 1994). The columella functions in the storage of mucilage, a complex carbohydrate that is synthesized and stored during seed development and secreted upon imbibition (Goto, 1982). We determined that *ap2* seed are also defective in mucilage synthesis or storage (B.G.W. den Boer, M. Edgar, and K.D. Jofuku, unpublished results) and that both normal seed coat morphology and mucilage production can be restored to *ap2-1* and *ap2-10* seed by introducing a single copy of the wild-type *AP2* gene into mutant plants (Figure 2; B.G.W. den Boer, unpublished results). Consistent with these observations, *AP2* is expressed in ovules (Figure 7) and in developing seed (B.G.W. den Boer, unpublished results).

AP2 together with at least three other genes, *TRANSPARENT TESTA*, *GLABRA* (*TTG*), *GLABROUS2* (*GL2*), and *ABERRANT TESTA SHAPE* (*ATS*), govern seed coat epidermal cell structure and mucilage production (Koornneef, 1981; Goto 1982; Léon-Kloosterziel et al., 1994). However, *ttg*, *gl2*, and *ats* flowers are phenotypically normal. Recent gene transformation experiments showed that the pleiotropic effects of *ttg* were complemented by introduction of the maize *R* gene (Lloyd et al., 1992), which encodes a plant homolog of the mammalian transcription factor *L-myc* (Ludwig et al., 1989). The relationship between *AP2* and *TTG* functions during seed coat development is not yet known.

AP2 Gene Expression in Vegetative Organs

Our finding that *AP2* is expressed at low levels as a polysomal mRNA in both the stem and vegetative leaf (Figure 6) was unexpected because there are no known reports of *ap2* mutations affecting vegetative development under standard growth conditions (Haughn and Somerville, 1988; Komaki et al., 1988; Bowman et al., 1989, 1991a; Kunst et al., 1989; K.D. Jofuku, B.G.W. den Boer, and J.K. Okamuro, unpublished observations). We confirmed that *AP2* was expressed in these tissues by showing that mutant gene transcripts were distinguishable as truncated mRNAs in *ap2-10* stem and leaf (Figure 6). We independently confirmed that *AP2* is transcriptionally active in leaf and stem using a chimeric reporter gene assay (B.G.W. den Boer and M. Van Montagu, unpublished results).

There are several hypotheses to explain why vegetative development is not affected in *ap2* mutants under standard growth conditions. First, *AP2* may not be required for vegetative growth

This content downloaded from 164.67.109.135 on Thu, 17 Jul 2014 21:23:12 PM
All use subject to JSTOR Terms and Conditions

and development. Some recent examples of regulatory genes whose expression has been detected in cells where they have no apparent genetic function include *LFY* in Arabidopsis (Weigel et al., 1992) and *unc-86* in *Caenorhabditis elegans* (Finney and Ruvkun, 1990). Alternatively, Arabidopsis may be genetically redundant for *AP2* function in stem and leaf. Low-stringency DNA gel blots and cDNA cloning experiments indicate that *AP2* belongs to a divergent multigene family (K.D. Jofuku and J.K. Okamuro, unpublished results). We and others have recently isolated several *AP2*-like genes from Arabidopsis; at least one of these genes is expressed in leaf (K.D. Jofuku and J.K. Okamuro, unpublished results). Finally, the effects of *ap2* mutations on vegetative development may have been masked by the choice of growth conditions. Consistent with this hypothesis, we recently found that *ap2-1* vegetative growth can be inhibited when compared to wild-type Arabidopsis Landsberg *erecta* (Ler) by specific soil conditions (K.D. Jofuku, unpublished results).

In conclusion, *AP2* belongs to a complex system of genes that regulate Arabidopsis flower initiation and development. Many genes in this regulatory network have recently been cloned and characterized. AP2 represents the newest class of plant floral homeotic proteins to be identified. Unlike other Arabidopsis floral meristem or organ identity genes, *AP2* is expressed throughout most of Arabidopsis development. Moreover, the pattern of *AP2* gene expression and gene regulation does not conform to the pattern predicted from earlier studies. Together, our results suggest that *AP2* is situated genetically upstream of the major flower-specific homeotic genes that regulate Arabidopsis flower development.

METHODS

Plant Material

Wild-type Arabidopsis Landsberg *erecta* (Ler) and Columbia C24 were used in this study. *ap2-1* (Ler) seed were provided by M. Koornneef (Wageningen Agricultural University, Wageningen, The Netherlands). *ap2-5* and *ap2-7* seed (Col-0) were from G. Haughn (University of British Columbia, Vancouver, Canada). *ap2-10* (T10) is in the C24 genetic background. Wild-type alleles are given in uppercase italics, and mutant alleles are designated in lowercase italics.

All plants were grown at 22°C under a 16-hr-light/8-hr-dark photoperiod unless otherwise specified.

Seed Coat NaOCl Assay

Arabidopsis seed were exposed to 5% sodium hypochlorite (NaOCl) for 10 min, washed five times in sterile water, and scored for viability by plating on seed germination medium (Valvekens et al., 1988). Under these conditions, wild-type seed are brown and produce viable seedlings. By contrast, *ap2* mutant seed are bleached and fail to germinate.

Arabidopsis Plant Transformation and T-DNA Insertional Mutagenesis

Arabidopsis C24 root explants were transformed with the Ti plasmid vector pTam3 according to the procedure of Valvekens et al. (1988). pTam3 contains the plant transposable element *Tam3* provided by C. Martin (John Innes Institute, Norwich, U.K.) cloned into the Ti plasmid vector pGSFR161 (De Block et al., 1987). pGSFR161 contains the plant selectable marker genes neomycin phosphotransferase II (*NPTII*) and phosphinothricin acetyltransferase (*BAR*) that confer resistance to the antibiotic kanamycin and the herbicide Basta (Hoechst, Frankfurt, Germany), respectively. *Tam3* was cloned into the *BAR* gene 5′ untranslated region so that transposon excision could be screened for by herbicide resistance (K.D. Jofuku, unpublished results). The *ap2-10* (T10) flower mutant was identified in a limited screen of independently transformed Arabidopsis lines and was found to be both kanamycin resistant and herbicide sensitive. DNA sequence analysis of the *ap2-10* gene showed that the mutation resulted from T-DNA insertion and not by *Tam3* excision and integration (data not shown).

Genetic Analyses

Reciprocal crosses between *ap2-1* and *ap2-10* (T10) plants revealed that the two mutations are allelic. Genetic and molecular analyses of *ap2-10* F_1 progeny revealed two unlinked T-DNA insertions in the *ap2-10* genome. We isolated a kanamycin-resistant plant that was heterozygous for *ap2-10* and possessed only one T-DNA insert after backcrossing once with wild-type Arabidopsis C24. Kanamycin-resistant, phenotypically wild-type progeny from this plant were used as pollen donors in a test cross with homozygous *ap2-1* mutants to score for recombination between T-DNA and *ap2-10* in the F_1 generation. We obtained and germinated ~2000 F_1 seed, selected 1000 kanamycin-resistant F_1 plants, and scored these for flower phenotype. All kanamycin-resistant F_1 plants selected showed a mutant floral phenotype indicating that the T-DNA insertion and *ap2-10* were genetically inseparable. We calculated the maximum genetic distance between the two loci (*r*, in centimorgans [cM]) using the following rationale: if T-DNA and *ap2-10* are genetically separable, then the probability of obtaining a recombinant gamete is equivalent to the frequency of recombination between the two loci (in percent)/2. Because we found no evidence of recombination between T-DNA and *ap2-10* in 1000 kanamycin-resistant F_1 plants and assuming that 1% recombination is equivalent to a genetic distance of 1 cM, then the maximum genetic distance between T-DNA and *ap2-10* (*r*) is less than or equal to the frequency of recombination (in percent)/2 or [(1/1000) × 100]/2 or 0.1 cM (Koornneef and Stam, 1992). If we then assume that 1 cM in the Arabidopsis genome represents ~180 kb, based on a genome size of 100,000 kb (Hauge and Goodman, 1992) and 553 cM (Koornneef, 1994), then the maximum genetic distance between T-DNA and *ap2-10* is less than 18 kb.

Scanning Electron Microscopy

Arabidopsis seeds were dried at 29°C for 3 to 5 days, mounted, and sputter coated with gold. Specimens were examined in a scanning electron microscope (JEOL Ltd., Tokyo, Japan) with an accelerating voltage of 10kV.

This content downloaded from 164.67.109.135 on Thu, 17 Jul 2014 21:23:12 PM
All use subject to JSTOR Terms and Conditions

DNA Isolation and Labeling

Arabidopsis total DNAs were isolated as described by Dellaporta et al. (1983) and were further purified by CsCl banding. Plasmid and phage DNAs were isolated as outlined by Jofuku and Goldberg (1988) and purified by anion exchange chromatography (Qiagen, Chatsworth, CA). DNAs were labeled using random oligonucleotides under conditions specified by Amersham.

Polysomal mRNA Isolation

Polysomal poly(A) mRNAs from Arabidopsis flowers, rosette leaves, and inflorescence stem internodes were isolated according to procedures described by Cox and Goldberg (1988).

Cloning and Sequence Analysis of AP2

Arabidopsis Ler and *ap2-10* (T10) genome libraries were generated in λ Charon 35 (Loenen and Blattner, 1983) using Sau3A partially digested total DNA from wild-type and mutant plants, respectively. A 30.2-kb T-DNA–containing region was assembled from overlapping genomic clones isolated from the *ap2-10* (T10) genome library. To localize the *APETALA2* (*AP2*) gene within this region, a corresponding wild-type *AP2* gene region was isolated from the Arabidopsis Ler genome library as a series of overlapping genomic clones, recloned in segments into the Ti plasmid vectors pGSFR161 (De Block et al., 1987) and pDE1000 (Denecke et al., 1992), and subsequently introduced into Arabidopsis *ap2-1* root explants via Agrobacterium-mediated transformation (Valvekens et al., 1988).

The wild-type *AP2* gene region was used to screen an Arabidopsis flower cDNA library (de Oliveira et al., 1993). Eleven clones corresponding to *AF2* were isolated from a screen of 220,000 clones. DNA sequence analysis suggests that *AF2* corresponds to an inosine-5′-monophosphate dehydrogenase (data not shown). Five clones corresponding to *AP2* were isolated. Clones λAP2c1, λAP2c5, and λAP2c3 contained 1.68-, 0.976-, and 0.447-kb cDNA inserts, respectively, which were then recloned into pGEM3 (Promega) and sequenced on both DNA strands by dideoxy methods using Sequenase II according to the manufacturer's specifications (U.S. Biochemicals). To determine the *AP2* gene sequence, the 7.2-kb EcoRI fragment described in Figure 2 was recloned into pGEM3 and sequenced on both strands using a series of oligonucleotide primers.

Nucleotide and Amino Acid Sequence Comparisons

Nucleotide and amino acid sequence comparisons were initially performed using the FASTDB software program (IntelliGenetics, Mountain View, CA). Amino acid sequence comparisons were also performed using the BLASTP program (Altschul et al., 1990) and default parameter settings to search a merged, nonredundant collection of sequences derived from the PIR 37.0, Swiss-Prot 26.0, and translated GenBank 78.0 data bases. Secondary structure predictions were based on the principles and software programs reviewed by Fasman (1989).

RNA and DNA Gel Blot Analyses

RNA and DNA gel blot studies were performed according to previously published procedures (Jofuku and Goldberg, 1988, 1989) using nylon membranes with modifications. DNA gel blot hybridizations were performed in 0.25 M $NaPO_4$, 7% SDS, 1% BSA, pH 7.2, at 65°C for 48 hr as described by Church and Gilbert (1984). RNA gel blot hybridizations were performed as specified by the manufacturer (Amersham). mRNA sizes were estimated relative to known RNA standards (Bethesda Research Laboratories). *AP2* mRNA prevalence in Arabidopsis flowers was estimated based on the frequency of *AP2* clones in a flower poly(A) RNA cDNA library.

In Situ Hybridization

Arabidopsis inflorescences were removed, fixed in 3.7% formaldehyde, 5% acetic acid, 50% ethanol, dehydrated, cleared in 10% chloroform in xylene, and embedded in paraffin essentially as described by O'Brien and McCully (1981) and Johansen (1940). In situ hybridization experiments with paraffin-embedded floral buds were performed as described by Cox and Goldberg (1988), with the exception that tissue sections were attached to microscope slides treated with 3-aminopropyltriethoxysilane (Sigma) (Silverthorne and Tobin, 1990). The *AP2* anti-mRNA and mRNA control probes were ^{35}S-labeled RNA probes synthesized from sequences within the AP2c1 plasmid subclone using the pGEM transcription system (Promega). The *AP2* anti-mRNA probe contained nucleotides 1 to 1371 of the *AP2* sequence shown in Figure 4. We showed previously in control mRNA gel blot studies that this probe is specific for *AP2* mRNA under similar hybridization conditions (data not shown). The *AP2* mRNA control probe included nucleotides 1 to 322 of the *AP2* sequence shown in Figure 4 and was used to assess background hybridization.

ACKNOWLEDGMENTS

We express our appreciation to Tracy Ferea for RNA expression analysis of *AP2* in wild-type and mutant plants; Ben Chu for isolation of the *ap2-5* and *ap2-7* mutant genes; Wilson Ardilles, Jan Gielen, and Nino Villaroel for DNA sequencing; and Kelly Matsudaira, Phyllis Strickland, and Drs. John Harada and Jane Silverthorne for advice regarding in situ hybridization. We are grateful to Rita Van Driessche, Wilfred Bentham, and Dr. Jonathan Krupp for their assistance with light microscopy and scanning electron microscopy and Linda Locatelli and Jim Velzy for excellent care of our plants. We thank Drs. George Haughn and Maarten Koornneef for providing *ap2* mutant seed and Drs. Dulce de Oliveira, Johan Botterman, Jürgen Denecke, and Cathie Martin for gifts of the Arabidopsis C24 flower cDNA library, pGSFR161, pDE1000, and pJAM4, respectively. We express our appreciation and thanks to Drs. Maarten Koornneef and Piet Stam for their invaluable advice regarding Arabidopsis, Drs. Robert Goldberg and Gary Drews for critical reading of this manuscript, and Dr. Saira Mian for sharing ideas on the *AP2* sequence. Finally, we wish to thank Drs. Robert Goldberg, Maarten Koornneef, Mieke Van Lijsebettens, Enno Krebbers, and Jan Leemans for their continued support. This study was supported by National Institutes of Health (NIH) Grant Nos. GM46309 and RR08132 to J.K.O. and funds from the University of California-Santa Cruz. Support

This content downloaded from 164.67.109.135 on Thu, 17 Jul 2014 21:23:12 PM
All use subject to JSTOR Terms and Conditions

for studies done in Belgium was provided by the Vlaams Actieprogramma Biotechnologie (VIAB), the Services of the Prime Minister (UIAP), and a European Community Bridge Grant No. BIOT-CT92-0529 to M.V.M. B.d.B. was supported by a European Economic Community predoctoral fellowship. K.D.J. was supported by National Science Foundation/North Atlantic Treaty Organization and EMBO Long-Term Postdoctoral fellowships while in Belgium. J.K.O. was an NIH postdoctoral fellow while in Belgium.

Received April 18, 1994; accepted June 27, 1994.

REFERENCES

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.

Bowman, J.L. (1992). Making cauliflower out of *Arabidopsis*: The specification of floral meristem identity. Flowering Newsletter **14,** 7–19.

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

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

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.

Chelsky, D., Ralph, R., and Jonak, G. (1989). Sequence requirements for synthetic peptide-mediated translocation to the nucleus. Mol. Cell. Biol. **9,** 2487–2492.

Church, G.M., and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA **81,** 1991–1995.

Coen, E.S., and Carpenter, R. (1993). The metamorphosis of flowers. Plant Cell **5,** 1175–1181.

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

Cox, K.H., and Goldberg, R.B. (1988). Analysis of plant gene expression. In Plant Molecular Biology: A Practical Approach, C.H. Shaw, ed (Oxford: IRL Press), pp. 1–35.

De Block, M., Botterman, J., Vandewiele, M., Dockx, J., Thoen, C., Gossele, V., Movva, N., Thompson, C., Van Montagu, M., and Leemans, J. (1987). Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J. **6,** 2513–2518.

de Oliveira, D.E., Franco, L.O., Simoens, C., Seurinck, J., Coppieters, J., Botterman, J., and Van Montagu, M. (1993). Inflorescence-specific genes from *Arabidopsis thaliana* encoding glycine-rich proteins. Plant J. **3,** 495–507.

Dellaporta, S.L., Wood, J., and Hicks, J.B. (1983). A plant DNA minipreparation: Version II. Plant Mol. Biol. Reporter **1,** 19–21.

Denecke, J., De Rycke, R., and Botterman, J. (1992). Plant and mammalian sorting signals for protein retention in the endoplasmic reticulum contain a conserved epitope. EMBO J. **11,** 2345–2355.

Dingwall, C., and Laskey, R.A. (1986). Protein import into the cell nucleus. Annu. Rev. Cell Biol. **2,** 367–390.

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

Drews, G.N., Weigel, D., and Meyerowitz, E.M. (1991b). Floral patterning. Curr. Opin. Genet. Dev. **1,** 174–178.

Fasman, G.D. (1989). Prediction of Protein Structure and the Principles of Protein Conformation. (New York: Plenum Press).

Finney, M., and Ruvkun, G. (1990). The *unc-86* gene couples cell lineage and cell identity in *C. elegans.* Cell **63,** 895–905.

Flemington, E., and Speck, S.H. (1990). Evidence for coiled-coil dimer formation by an Epstein-Barr virus transactivator that lacks a heptad repeat of leucine residues. Proc. Natl. Acad. Sci. USA **87,** 9459–9463.

Garcia-Bustos, J., Heitman, J., and Hall, M.N. (1991). Nuclear protein localization. Biochem. Biophys. Acta **1071,** 83–101.

Goto, N. (1982). The relationship between characteristics of seed coat and dark germination by gibberellin. Arabidopsis Info. Serv. **19,** 29–34.

Gustafson-Brown, C., Savidge, B., and Yanofsky, M.F. (1994). Regulation of the Arabidopsis floral homeotic gene *APETALA1.* Cell **76,** 131–143.

Hauge, B.M., and Goodman, H.M. (1992). Genome mapping in Arabidopsis. In Methods in Arabidopsis Research, C. Koncz, N.-H. Chua, and J. Schell, eds (Singapore: World Scientific), pp. 191–223.

Haughn, G.W., and Somerville, C.R. (1988). Genetic control of morphogenesis in Arabidopsis. Dev. Genet. **9,** 73–89.

Howard, E.A., Zupan, J.R., Citovsky, V., and Zambryski, P.C. (1992). The VirD2 protein of *A. tumefaciens* contains a C-terminal bipartite nuclear localization signal: Implications for nuclear uptake of DNA in plant cells. Cell **68,** 109–118.

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

Hunter, T., and Karin, M. (1992). The regulation of transcription by phosphorylation. Cell **70,** 375–387.

Irish, V.F., and Sussex, I.M. (1990). Function of the *apetala-1* gene during *Arabidopsis* floral development. Plant Cell **2,** 741–753.

Jack, T., Brockman, L.L., and Meyerowitz, E.M. (1992). The homeotic gene *APETALA3* of *Arabidopsis thaliana* encodes a MADS box and is expressed in petals and stamens. Cell **68,** 683–697.

Jofuku, K.D., and Goldberg, R.B. (1988). Analysis of plant gene structure. In Plant Molecular Biology: A Practical Approach, C.H. Shaw, ed (Oxford: IRL Press), pp. 37–66.

Jofuku, K.D., and Goldberg, R.B. (1989). Kunitz trypsin inhibitor genes are differentially expressed during the soybean life cycle and in transformed tobacco plants. Plant Cell **1,** 1079–1093.

Johansen, D.A. (1940). Plant Microtechnique (New York: McGraw-Hill Book Co., Inc.).

Jones, M.K., Anantharamaiah, G.M., and Segrest, J.P. (1992). Computer programs to identify and classify amphipathic and α-helical domains. J. Lipid Res. **33,** 287–296.

Komaki, M.K., Okada, K., Nishino, E., and Shimura, Y. (1988). Isolation and characterization of novel mutants of *Arabidopsis thaliana* defective in flower development. Development **104,** 195–203.

Koornneef, M. (1981). The complex syndrome of TTG mutants. Arabidopsis Info. Serv. **18,** 45–51.

This content downloaded from 164.67.109.135 on Thu, 17 Jul 2014 21:23:12 PM
All use subject to JSTOR Terms and Conditions

Koornneef, M. (1994). Arabidopsis genetics. In Arabidopsis, E. Meyerowitz, and C. Somerville, eds (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), in press.

Koornneef, M., and Stam, P. (1992). Genetic analysis. In Methods in Arabidopsis Research, C. Koncz, N.-H. Chua, and J. Schell, eds (Singapore: World Scientific), pp. 83–99.

Koornneef, M., de Bruine, J.-H., and Goettsch, P. (1980). A provisional map of chromosome 4 of *Arabidopsis*. Arabidopsis Info. Serv. **17,** 11–18.

Kunst, L., Klenz, 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.

Léon-Kloosterziel, K.M., Keijzer, C.J., and Koornneef, M. (1994). A seed shape mutant of Arabidopsis that is affected in integument development. Plant Cell **6,** 385–392.

Lloyd, A.M., Walbot, V., and Davis, R.W. (1992). *Arabidopsis* and *Nicotiana* anthocyanin production activated by maize regulators *R* and *C1*. Science **258,** 1773–1775.

Loenen, W.A.M., and Blattner, F.B. (1983). Lambda Charon vectors (Ch 32, 33, 34 and 35) adapted for DNA cloning in recombinant-deficient hosts. Gene **26,** 171–179.

Ludwig, S.R., Habera, L.F., Dellaporta, S.L., and Wessler, S.R. (1989). *Lc*, a member of the maize *R* gene family responsible for tissue-specific anthocyanin production, encodes a protein similar to transcriptional activators and contains the *myc*-homology region. Proc. Natl. Acad. Sci. USA **86,** 7092–7096.

Ma, H. (1994). The unfolding drama of flower development: Recent results from genetic and molecular analyses. Genes Dev. **8,** 745–756.

Ma, H., Yanofsky, M.F., and Meyerowitz, E.M. (1991). *AGL1–AGL6*, an *Arabidopsis* gene family with similarity to floral homeotic and transcription factor genes. Genes Dev. **5,** 484–495.

Mandel, M.A., Bowman, J.L., Kempin, S.A., Ma, H., Meyerowitz, E.M., and Yanofsky, M.F. (1992a). Manipulation of flower structure in transgenic tobacco. Cell **71,** 133–143.

Mandel, M.A., Gustafson-Brown, C., Savidge, B., and Yanofsky, M.F. (1992b). Molecular characterization of the *Arabidopsis* floral homeotic gene *APETALA1*. Nature **360,** 273–277.

Meyerowitz, E.M., Bowman, J.L., Brockman, L.L., Drews, G.N., Jack, T., Sieburth, L., and Weigel, D. (1991). A genetic and molecular model for flower development in *Arabidopsis thaliana*. Development Suppl. **1,** 157–167.

Mitchell, P.J., and Tjian, R. (1989). Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245,** 371–378.

Mizukami, Y., and Ma, H. (1992). Ectopic expression of the floral homeotic gene *AGAMOUS* in transgenic Arabidopsis plants alters floral organ identity. Cell **71,** 119–131.

Modrusan, Z., Reiser, L., Feldmann, K.A., Fischer, R.L., and Haughn, G.W. (1994). Homeotic transformation of ovules into carpel-like structures in Arabidopsis. Plant Cell **6,** 333–349.

O'Brien, T.P., and McCully, M.E. (1981). The Study of Plant Structure: Principles and Selected Methods. (Melbourne: Termarcarphi Pty. Ltd.).

Okamuro, J.K., den Boer, B.G.W., and Jofuku, K.D. (1993). Regulation of Arabidopsis flower development. Plant Cell **5,** 1183–1193.

Schultz, E.A., and Haughn, G.W. (1993). Genetic analysis of the floral initiation process (FLIP) in *Arabidopsis*. Development **119,** 745–765.

Segrest, J.P., De Loof, H., Dohlman, J.G., Brouillette, C.G., and Anantharamaiah, G.M. (1990). Amphipathic helix motif: Classes and properties. Proteins **8,** 103–117.

Shannon, S., and Meeks-Wagner, D.R. (1993). Genetic interactions that regulate inflorescence development in Arabidopsis. Plant Cell **5,** 639–655.

Shiraishi, H., Okado, K., and Shimura, Y. (1993). Nucleotide sequences recognized by the AGAMOUS MADS domain of *Arabidopsis thaliana in vitro*. Plant J. **4,** 385–398.

Silverthorne, J., and Tobin, E.M. (1990). Post-transcriptional regulation of organ-specific expression of individual *rbc*S mRNAs in *Lemna gibba*. Plant Cell **2,** 1181–1190.

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

Valvekens, D., Van Montagu, M., and Van Lijsebettens, M. (1988). *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis* root explants using kanamycin selection. Proc. Natl. Acad. Sci. USA **85,** 5536–5540.

van der Krol, A.R., and Chua, N.-H. (1993). Flower development in petunia. Plant Cell **5,** 1195–1203.

Van Lijsebettens, M., Vanderhaeghen, R., and Van Montagu, M. (1991). Insertional mutagenesis in *Arabidopsis thaliana:* Isolation of a T-DNA linked mutation that alters leaf morphology. Theor. Appl. Genet. **2,** 277–284.

Varagona, M.J., Schmidt, R.J., and Raikhel, N.V. (1992). Nuclear localization signal(s) required for nuclear targeting of the maize regulatory protein Opaque-2. Plant Cell **4,** 1213–1227.

Veit, B., Schmidt, R.J., Hake, S., and Yanofsky, M.F. (1993). Maize floral development: New genes and old mutants. Plant Cell **5,** 1205–1215.

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

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.

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.

This content downloaded from 164.67.109.135 on Thu, 17 Jul 2014 21:23:12 PM
All use subject to JSTOR Terms and Conditions