RESEARCH ARTICLE

Petunia Ap2-like Genes and Their Role in Flower and Seed Development

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We have isolated three Apetala2 (Ap2)-like genes from petunia and studied their expression patterns by in situ hybridization. PhAp2A has a high sequence similarity to the A function gene Ap2 from Arabidopsis and a similar expression pattern during flower development, suggesting that they are cognate orthologs. PhAp2B and PhAp2C encode for AP2-like proteins that belong to a different subgroup of the AP2 family of transcription factors and exhibit divergent, nearly complementary expression patterns during flower development compared with PhAp2A. In contrast, all three PhAp2 genes are strongly expressed in endosperm. The phenotype of the petunia A-type mutant blind cannot be attributed to mutations in the petunia Ap2 homologs identified in this study, and reverse genetics strategies applied to identify phap2a mutants indicate that PhAp2A might not be essential for normal perianth development in petunia. Nevertheless, we show that PhAp2A is capable of restoring the homeotic transformations observed in flowers and seed of the ap2-1 mutant of Arabidopsis. Although the interspecific complementation proves that PhAp2A encodes a genuine Ap2 ortholog from petunia, additional factors may be involved in the control of perianth identity in this species.

INTRODUCTION

The overall conservation of flower developmental mechanisms in different species is illustrated by several observations. First, analogous homeotic mutants exist in different species, and generally their phenotypes result from mutations in homologous genes. Analysis of homeotic flower organ mutants has resulted in the ABC model, which proposes that three types of functions (A, B, and C) exist that determine the identity of developing primordia in a combinatorial manner (Coen and Meyerowitz, 1991).

Second, changes in the expression pattern of a C function gene in a given species often have phenotypic consequences comparable to similar alterations in the expression pattern of its homolog in other species (Mizukami and Ma, 1992; Bradley et al., 1993; Tsuchimoto et al., 1993; Pnueli et al., 1994).

Third, flower developmental genes can be conserved to the extent that their products are functional across species barriers. Overexpression of flower meristem and organ development genes can cause similar phenotypic changes in various species (Mandel et al., 1992; Weigel and Nilsson, 1995), and B-type mutants have been complemented by the introduction of B-type genes from other species (Samach et al., 1997). No data have been reported that allow a comparison of the function of the homologs of the A-class gene Apetala2 (Ap2) among the different model systems.

At an early stage of flower development, the Ap2 gene of Arabidopsis promotes the establishment of the floral meristem, in cooperation with Apetala1 (Ap1), Leafy (Lfy), and Cauliflower1 (CAL1; Irish and Sussex, 1990; Bowman et al., 1993). During floral development, Ap2 is essential for the determination of the identity of sepals and petals. In the weak ap2-1 mutant, leaf-like organs replace sepals, and petals exhibit antheroid characteristics; in strong ap2 mutants, carpels are formed in the outer whorl of the flower, petals are absent, and the number of stamens is reduced (Komaki et al., 1988; Kunst et al., 1989). The homeotic transformations found in ap2 mutants can be explained in part by ectopic expression of Agamous (Ag), as observed in the two outer whorls of their flowers (Drews et al., 1991). Apart from Ap2, two other genes, Leunig (Lug) and Curly Leaf (Clf), are involved in the repression of Ag in the perianth (Liu and Meyerowitz, 1995; Goodrich et al., 1997). Ap2 also is required for normal seed

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Figure 1. DNA Gel Blot Analysis of \textit{PhAp2} Genes in Petunia (Line V23) and Amino Acid Alignments of Petunia \textit{PhAp2} genes and Arabidopsis \textit{Ap2}.
coat development, and ap2 mutants lack the epidermal plateau of the epidermis of wild-type seed (Jofuku et al., 1994; Leon-Kloosterziel et al., 1994; Modrusan et al., 1994).

Ap2 was isolated by T-DNA tagging. It encodes a protein that belongs to a rapidly growing family of plant-specific transcription factors that contains the AP2 domain, a conserved ~60–amino acid region involved in DNA binding (Okamuro et al., 1997; Riechman and Meyerowitz, 1998). The AP2 family of proteins consists of two main branches. One branch contains proteins that have a single copy of the AP2 domain. These are involved in different kinds of stress responses and include the ethylene responsive element AP2 domain. These are involved in different kinds of stress responses and include the ethylene responsive element binding proteins and the C repeat binding factor CBF1 (Ohme-Takagi and Shinshi, 1995; Stockinger et al., 1997).

Ap2, Antegumenta (Ant), Glossy15 (Gl15), and Indeterminate spikelet 1 (Ids1) all contain two AP2 domains and belong to the second branch of the AP2-like transcription factor family that plays a role in plant development. Ant is required for ovule and female gametophyte development (Elliott et al., 1996; Klucher et al., 1996), Gl15 is required for the maintenance of juvenile traits of epidermal cells in maize leaves from node 2 to node 6 (Moose and Sisco, 1996), and Ids1 controls maize spikelet meristem fate (Chuck et al., 1998).

In this study, we report the isolation and partial characterization of three AP2-like genes of petunia: PhAp2A, PhAp2B, and PhAp2C. To determine which of these three genes represents the Ap2 ortholog in petunia, we compared their sequences with Ap2, analyzed their expression patterns during plant development by in situ hybridization, and applied a reverse genetics approach to identify mutant alleles by transposon insertion mutagenesis. We also mapped the three gene copies to determine if one of them could encode Blind (Bl), which appeared not to be the case. This A-type mutant from petunia has been described previously and has been suggested to be caused by the loss of activity of the petunia Ap2 ortholog (Vallade et al., 1987; Tsuchimoto et al., 1993). The phenotype of flowers of the Bl mutant is similar to that seen in ap2 and clf mutants in Arabidopsis and in fusiata (fis) mutants in Antirrhinum species (Motte et al., 1998).

In addition, we have demonstrated the functional conservation of the A-type genes PhAp2A and Ap2 by complementing the ap2-1 mutant of Arabidopsis with the petunia PhAp2A gene.

RESULTS


An 800-bp PhAp2A-1 cDNA insert was used to probe petunia genomic DNA gel blots at medium stringency. Under these conditions, three hybridizing bands can be observed, one of which has a higher intensity than the other two (Figure 1A). When the blot was stripped and rehybridized with a 4-kb PhAp2A genomic subclone, only the strongest hybridizing band was observed (Figure 1B). These results indicate that PhAp2A belongs to a gene family and that at least two other PhAp2A homologous gene copies exist in the petunia genome, which were termed PhAp2B and PhAp2C.

The PhAp2A cDNA encodes a protein of 519 amino acid residues (Figure 1C). Similarity searches using tBlastn revealed the Arabidopsis AP2 protein as the most homologous entry in the GenBank database. PHAP2A shows 64% overall amino acid identity and 77% similarity with AP2. The homology of PHAP2A and AP2 is not limited to the AP2 domains but extends through the reading frame of the two proteins. A serine-rich putative transcription activation domain (amino acids 18 to 60) and a putative nuclear localization signal (amino acids 148 to 158; Jofuku et al., 1994) are conserved, as are the N and C termini of the proteins (Figure 1C). The homology between the two proteins is especially high in the two AP2 domains (63 of 67 and 66 of 68 residues identical), whereas the linker that connects them is completely conserved at the amino acid level (Figure 1C).

The PhAp2B cDNA encodes a 457–amino acid protein (Figure 1C). The AP2 domains and the linker of PHAP2A and AP2 are more closely related than those of the two petunia proteins PHAP2A and PHAP2B. Interestingly, of the 21 amino acid substitutions that occur in this region between PHAP2A and PHAP2B, five amino acids are similarly substituted in the two domains and two more in the linker of the predicted Arabidopsis RAP2.7 sequence (Figure 1C).

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Figure 1. (continued).
(A) Blot probed with the 800-bp PhAp2A-1 cDNA fragment that contains the Ap2 domains (medium stringency).
(B) Blot probed with the 4-kb genomic XbaI fragment of PhAp2A (high stringency).
(C) Comparison of the overall amino acid sequences, the AP2 domains (shaded), linkers, and putative nuclear localization signals (in boldface) of PhAp2A (GenBank accession number AF132001), Ap2 (ATU12546), PhAp2B (AF132002), PhAp2C (partial sequence), RAP2.7 (AF003100), and AN7 (U40256). Amino acids in the AP2 domains, which are conserved in all mentioned sequences, are in white on a shaded background; the five conserved amino acids in the domains of PhAp2B and RAP2.7 are white on black. Gaps are indicated by dashes. No putative nuclear localization signal could be identified in front of the first AP2 domain in the Ant gene; the overall homology of Rap2.7 and Ant with the other AP2-like genes outside the domain regions was too low to allow a meaningful comparison.
Figure 2. In Situ Analysis of Expression of PhAp2A, PhAp2B, PhAp2C, and pMADS3 during Flower Development.

Each row represents a set of consecutive 10-μm sections that were hybridized with the different 35S-CTP-labeled antisense riboprobes (except for [E], which represents a similar section).
Outside of the AP2 domains, there is very little sequence conservation between PHAP2A and PHAP2B. Two conserved motifs (F/VDF/ILKT/V and VTKEF/LFPV/LS) could be identified in the N-terminal part of PHAP2B and PHAP2C and in the Arabidopsis RAP2.7 (GenBank accession number T20443; Okamuro et al., 1997), and another one could be identified in the C-terminal part of PHAP2B and RAP2.7 (PL/MFSV/TAAASSGF). These data suggest that PHAP2B, PHAP2C, and RAP2.7 belong to a different subgroup than AP2 and PHAP2A (Figure 1C).

No PhAp2C cDNA clone could be retrieved from the inflorescence or ovary cDNA library, although reverse transcription–polymerase chain reaction (RT-PCR) using two PhAp2C-specific primers on young petunia inflorescences and flowers suggested that the gene is expressed.

Chromosomal Localization of PhAp2A, PhAp2B, and PhAp2C Shows That They Do Not Coincide with the Bl Locus

Flowers of the bl mutant of petunia have patches of stigmoid tissue at the tips of their sepals, and antheroids replace the flower limb. These homeotic transformations are caused by ectopic expression of the C-type gene pMADS3 (pMADS3 = FBP14; Kater et al., 1998) in bl leaves, sepals, and petals (Tsuchimoto et al., 1993). It has been proposed that Bl is a negative regulator of pMADS3 and that Bl determines the expression boundaries of pMADS3 (Tsuchimoto et al., 1993). Because Ap2 is a negative regulator of the C-type gene Ag in Arabidopsis, we wanted to determine if one of its homologs in petunia coincides with the Bl locus.

We performed a linkage analysis for the three PhAp2 genes by analyzing restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP; Vos et al., 1995), and phenotypic markers in different backcrosses. According to the (V26 × W137) × W137 progeny analysis, PhAp2A is located on chromosome 4, PhAp2B is located on chromosome 2, and PhAp2C is located on chromosome 6. PhAp2A and the Bl locus, both on chromosome 4, appear to be loosely linked (see Methods for details). Thus, none of the PhAp2 loci coincides with the Bl locus.

Expression of PhAp2 Genes during Plant Development

The spatial and temporal distribution of the PhAp2A, PhAp2B, and PhAp2C mRNA and the mRNA expression of the petunia C-type gene pMADS3 were analyzed by in situ hybridization of consecutive sections of wild-type inflorescences and flowers or flower parts (Figures 2A, 2E, 2I, and 2M) to 35S-labeled PhAp2A, PhAp2B, PhAp2C, and pMADS3 antisense riboprobes. The specificity of the probes used for the in situ hybridization experiments was tested by RNA gel blot analysis (not shown). The different stages of flower development referred to in the text are described in Table 1.


In the wild-type petunia inflorescence, uniform PhAp2A expression could be observed in stage 1 to 2 inflorescence

Figure 2. (continued).

(A) Bright-field photograph of the top of the inflorescence with a transverse section through the inflorescence meristem and a stage 4 to 5 flower and an oblique section through a stage 6 flower and part of a stage 7 flower.
(B) Dark-field photograph of a section like that shown in (A) with PhAp2A expression.
(C) Dark-field photograph of a section like that shown in (A) with PhAp2B expression.
(D) Dark-field photograph of a section like that shown in (A) with pMADS3 expression.
(E) Bright-field photograph of a longitudinal section through a stage 7 flower.
(F) Dark-field photograph of a section like that shown in (E) with PhAp2A expression.
(G) Dark-field photograph of a section like that shown in (E) with PhAp2B expression.
(H) Dark-field photograph of a section like that shown in (E) with pMADS3 expression.
(I) Bright-field photograph of a transverse section through a stage 8 to 9 flower.
(J) Dark-field photograph of a section like that shown in (I) with PhAp2A expression.
(K) Dark-field photograph of a section like that shown in (I) with PhAp2C expression.
(L) Dark-field photograph of a section like that shown in (I) with pMADS3 expression.
(M) Bright-field photograph of a longitudinal section through an inflorescence with an early stage 9 flower and a stage 6 flower.
(N) Dark-field photograph of a section like that shown in (M) with PhAp2A expression.
(O) Dark-field photograph of a section like that shown in (M) with PhAp2C expression.
(P) Dark-field photograph of a section like that shown in (M) with pMADS3 expression.

ac, connective tissue of the anther; ap, anther primordium; br, bract; ca, carpel; fp, flower primordium; ov, ovary; ow, ovary wall; pc, procambium; pe, petal; pl, placenta; se, sepal; st, stamen; vb, vascular bundle. Bars in (A), (E), (I), and (M) = 100 μm.
meristems and in young stage 4 to 5 flower buds. A weaker signal was observed in the procambial cells of the developing stem. In bracts subtending stage 4 to 5 flower buds and in stage 6 sepals, the PhAp2A mRNA was not distributed uniformly but localized to a layer of ground parenchyma cells (Figure 2B). The expression of PhAp2A in sepals and bracts decreased after stage 7 to 8 (Figures 2F, 2J, and 2N). Petals exhibited strong PhAp2A expression throughout their development. In young petal primordium until stage 5 to 6, the signal had a uniform appearance (Figure 2B). In later stages, PhAp2A was expressed in a layer of cells in the ground parenchyma, as in sepals and bracts. This layer runs through the center of the flower tube tissue, assumes a more adaxial position in the flower limb (Figures 2J and 2N), and passes around the dorsal side of the vascular bundle of the main vein of the petal (not shown).

Stamen primordia of stage 6 flower buds showed uniform expression that decreased by stage 7 and became concentrated in the region where the vascular bundles of the filaments develop (Figures 2B, 2F, and 2J). At stage 9, weak expression also was detected in the anther wall and in the connective tissue (not shown). A hybridizing signal also was detected over mature pollen grains (Figure 3H). Young carpel primordia of stage 7 flower buds exhibited strong PhAp2A expression (Figure 2F). In stage 7 to 9 flower buds, the signal was strong in the central cell layers of the ovary wall, in the placenta, and in ovule primordia (Figures 2J and 2N). When the ovules became stalked, the signal in the ovary wall and the placenta decreased. PhAp2A mRNA then could be detected mainly in the vascular bundle of the placenta, and a weak signal also could be seen in the vascular bundle of the style and stigma (Figure 3B). By stage 11, the hybridization signal in the ovules became restricted to the funiculus and the integument (Figures 3E and 3K).

PhAp2B and PhAp2C expression was detected in the outer cell layers of young bracts subtending stage 1 to 2 inflorescence meristems. Stage 7 flowers expressed PhAp2B and PhAp2C in the epidermis of the sepals (Figures 2C and 2G). Expression was maintained in sepals at least until stage 9 to 10, by which time the signal was no longer localized specifically in the outer cell layers and acquired a spotty appearance (Figures 2K and 2O). Weaker PhAp2B and PhAp2C expression also was detected in the outer cell layers around the main veins of stage 9 petals (Figure 2K). The two genes were strongly expressed in the connective tissue of stage 8 to 9 stamens and around stage 10 to 11 as well in the anther wall. No signal was observed over the vascular bundles and the filaments or, at this stage, over the developing microspores (Figures 2K, 2O, 3C, and 3D). However, in mature anther filaments, a spotty PhAp2C signal was observed (not shown), and although the connective tissue degenerated and the hybridization signal disappeared from the anther wall, PhAp2B and PhAp2C signals could be observed over the pollen grains (Figure 3I). The fourth whorl gynoecia of
stage 9 flowers exhibited low PhAp2B and PhAp2C expression. As the style elongated, the signal increased in intensity and became restricted to the epidermis of the ovary wall (Figures 2K, 3C, 3D, 3F, and 3L). We did not detect any PhAp2B or PhAp2C expression in the placenta or in the developing ovules during the stages of flower development described in this article.

The expression patterns of the PhAp2 genes were compared with the pattern of the C function gene pMADS3. pMADS3 expression first was detected in the center of stage 5 flowers, before any stamen primordia development was visible. The expression in developing stamen primordia remained uniform until stage 7, when the anthers and filaments start to differentiate (Figures 2D and 2H). Stage 8 to 9 stamens showed some pMADS3 expression in the anther wall and in the connective tissue, but the strongest signal was observed over the vascular bundle in the filament (Figures 2L and 2P). No signal was detected over the developing ovules during the stages of flower development described in this article.

The expression of the PhAp2 genes was investigated further in mature seed, germinating seedlings, and young plants. Strong expression was detected for PhAp2A, PhAp2B, and PhAp2C in the endosperm of mature seed. The hybridizing signal in endosperm was distributed asymmetrically, in that the side where the radicle emerges during germination did not show any expression (Figures 4A to 4D). No expression could be detected in the mature embryo. Leaf primordia of young seedlings showed a clear signal for PhAp2A, as did the leaf primordia and the leaves of young plantlets (Figures 4E to 4H).

The expression of the PhAp2A gene thus differs markedly from that of PhAp2B/PhAp2C during vegetative and floral development; in contrast, the expression patterns of the three genes coincide in mature seed. Mutants in which the functions of these genes have been abolished are required to reveal the roles of the PhAp2 genes during plant development.


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Transposon Insertion Mutagenesis of PhAp2A

Based on the results of in situ gene expression analysis and sequence comparisons, PhAp2A was the only likely candidate to perform the orthologous Atp2 function in petunia. To prove that PhAp2A functions as an A-type gene, we screened for loss-of-function alleles by transposon insertion mutagenesis. A PCR-based screening method (Ballinger and Benzer, 1989; Kaiser and Goodwin, 1990) was used to select for insertions of the nonautonomous transposable element dTph1 in PhAp2A by using three available petunia populations (Koes et al., 1995; Figure 5A). A set of PhAp2A-specific primers and a primer complementary to the terminal inverted repeat of the transposon were used; five heterozygous insertion mutants were detected in a population of 3456 W138 plants (Figure 5B).

The positions of the dTph1 elements were determined by cloning and sequencing of the fragments flanking the transposon insertions in PhAp2A. Three plants contained a dTph1 insertion in intron sequences (T2194-14, intron VIII; V2047-10, intron I; V2193-7, intron III). The two other plants harbored a dTph1 element in the first exon, after amino acids 17 (V2116-10) and 141 (V2025-6) (Figure 5B). None of the homozygous insertion mutants segregating in the progeny of the selfed heterozygous primary insertion mutants had an altered phenotype.

We used RT-PCR with two gene-specific primers flanking the exon 1 insertion site to analyze the PhAp2A mRNA in the exon 1 insertion mutants V2025-6 and V2116-10. The mutant cDNA fragments are ~300 bp longer than the wild-type PhAp2A cDNA fragments. This suggests that there is no premature termination of the messenger due to transcription termination signals in the element and that dTph1 is cotranscribed with the PhAp2A messenger (shown for the V2025-6 insertion allele in Figure 5C). Also, wild-type and mutant cDNA fragments exhibited a comparable abundance in plants heterozygous for the insertion. Thus, the dTph1 insertions did not seem to affect the level of PhAp2A transcription. The dTph1 element is 284 bp long and induces a target site duplication of 8 bp upon insertion. The translated nucleotide sequence of the elements inserted in the PhAp2A gene contains stop codons in the three reading frames. Thus, theoretically, only 17 amino acids of the PHAP2A protein of V2116-10 and 141 amino acids of the PHAP2A protein of V2025-6 would be translated, which should result in a functional knockout of the PhAp2A gene.

In plants homozygous for the V2025-6 insertion, a low level of wild-type mRNA was detected by RT-PCR. To determine whether this product was formed after excision of dTph1 or by alternative splicing, we crossed this insertion allele into an activator minus background to stabilize the insertion. The Activator element controls transposition of dTph1 elements (Gerats et al., 1990). Stabilization of the dTph1 system was monitored by means of a dTph1-tagged reporter gene that controls anthocyanin synthesis (see Methods). Plants homozygous for the PhAp2A(V2025-6) allele and lacking the active Activator produced only the...
Figure 3. Expression of PhAp2 Genes and pMADS3 in Third and Fourth Whorl Organs.
mutant mRNA and, again, exhibited a wild-type phenotype. The expression patterns of PhAp2A and pMADS3 in plants homozygous for the PhAp2A(V2025-6) insertion allele were analyzed by in situ hybridization and did not differ from the patterns found in wild-type petunia (results not shown).

**PhAp2A Complements the ap2-1 Mutant of Arabidopsis**

Because the phap2a insertion mutants we obtained did not exhibit a mutant phenotype, we decided to express PhAp2A in an ap2-1 background in Arabidopsis to determine whether this might result in complementation. The ap2-1 mutant is temperature sensitive. At 15°C, the sepals have leaf-like characteristics, and the petals are slightly phylloid; at 25°C, the sepals are stigmoid leaves, and the petals are staminoid; and at 29°C, the sepals are carpelloid, and the petals are absent, resembling the phenotype of the strong ap2 mutants (Bowman et al., 1989). The binary vector pBIN19-3SS PhAp2A was used to transform the ap2-1 mutant (see Methods). Kanamycin-resistant calli and shoots were selected at 23°C, but rooted transformants were allowed to develop at 26°C to induce a more extreme ap2-1 phenotype.

All shoots from four of five independent transformants generated phenotypically wild-type flowers that produced phenotypically wild-type seed (Figure 6). Together with the sequence and expression data, the complementation data prove that PhAp2A encodes the Ap2 ortholog of petunia.

**DISCUSSION**

In this study, we report the isolation and partial characterization of three Ap2-like genes from petunia: PhAp2A, PhAp2B, and PhAp2C. The PHAP2A protein is the closest AP2 homolog described so far. The 18–amino acid cores of the AP2 domain, which theoretically are capable of forming an amphipathic α helix (Jofuku et al., 1994), are identical in the two proteins. If the DNA binding specificity of the AP2-like proteins is determined by the amphipathic α-helix core itself, PHAP2A should be able to bind the target sequences of AP2. In contrast with what has been observed for other AP2-like proteins, the homology between PHAP2A and AP2 extends throughout the whole protein, suggesting that these genes are orthologs.

Two closely related Ap2-like gene copies, PhAp2B and PhAp2C, detected by DNA gel blot hybridization were isolated. Based on the occurrence of similar amino acid substitutions in their AP2 domains, PHAP2B and PHAP2C, like RAP2.7, and IDS1 belong to a different subgroup of AP2-like transcription factors than AP2 and PHAP2A.

**Expression Patterns of PhAp2A and PhAp2B/PhAp2C Suggest That These Genes May Have Both Overlapping and Unique Functions**

Like Ap2 and Ant in Arabidopsis, PhAp2A is expressed uniformly in young lateral organ primordia. As the organs mature, the signal in developing bracts, sepals, petals, and the ovary wall gradually decreases in the outer layers of these organs and eventually disappears.

During flower development, PhAp2B and PhAp2C expression patterns are remarkably complementary to that of PhAp2A. PhAp2B and PhAp2C expression begins to appear in bracts, sepals, petals, and the ovary wall when PhAp2A expression is decreasing. In general, we observed little if any overlap between the expression of PhAp2A and that of PhAp2B and PhAp2C. Only some of the Ap2-like genes that have been described have been well characterized in terms of both expression and function. The Gl15 gene has been described to control the identity of epidermal cells in maize (Moose and Sisco, 1996). The expression patterns of PhAp2B and PhAp2C are compatible with a function in the development of the...
epidermis. The divergence of the amino acid sequences between PHAP2B/PHAP2C and GL15, however, suggests that they are not orthologous.

PhAp2A also is expressed during vegetative development. A strong signal can be observed in leaf primordia of young seedlings that at later stages decreases and concentrates in the inner layers of the developing leaves. Thus, the progression of PhAp2A expression during floral development is the same as that during vegetative development, supporting the idea that the floral organs are nothing but specialized, modified leaves (von Goethe, 1790).

The homology between the AP2 protein of Arabidopsis and the PHAP2A protein of petunia, together with the resemblance of their expression patterns, strongly suggests that the two genes are genuine orthologs.

Like Ap2 and Ant of Arabidopsis, PhAp2A also is expressed during ovule development. The PhAp2A expression in integuments is in accordance with the role of Ap2 in seed

Figure 4. Expression of PhAp2 Genes in Germinating Seed and Expression of PhAp2A in Young Seedlings and Young Vegetative Plants.

(A) Phase-contrast photograph of a stained longitudinal section through a germinating seedling.
(B) Dark-field photograph of a section like that shown in (A) with PhAp2A expression.
(C) Dark-field photograph of a section like that shown in (A) with PhAp2B expression.
(D) Dark-field photograph of a section like that shown in (A) with PhAp2C expression.
(E) Bright-field photograph of a longitudinal section through a seedling with emerging leaf primordia.
(F) Dark-field photograph of a section like that shown in (E) with PhAp2A expression.
(G) Bright-field photograph of a longitudinal section through the apex of a young vegetative plantlet.
(H) Dark-field photograph of a section like that shown in (G) with PhAp2A expression.

co, cotyledon; en, endosperm; le, leaf; lp, leaf primordium; r, radicle. Bars in (A) to (H) = 100 μm.
coat development observed in Arabidopsis. PhAp2A, PhAp2B, and PhAp2C show an identical expression pattern in mature and germinating seed. A strong, asymmetrical signal is observed in the endosperm, in which it is excluded from the side of radicle emergence. This expression pattern resembles that of prolamin seed storage proteins of maize (also called zeins), which start to be expressed coordinately in the endosperm of maize 8 to 10 days after pollination. The promoters of these zein genes share a common regulatory element, the prolamin box (5'-TGTAAAG-3'), that is a good candidate for being the cis-acting regulatory element controlling this expression pattern (Forde et al., 1985). Recently, an endosperm-specific factor belonging to the Dof family of zinc finger proteins that binds the prolamin box and interacts with Opaque 2, a known transcriptional activator of zein gene expression, was isolated from maize (Vicente-Carbajosa et al., 1997). A perfect prolamin box (TGTTAAAG) and four related elements (TGGAAARK) were detected in the promoter of the PhAp2A gene (T. Maes, results not shown). At least one other Ap2-like gene, an Ant homolog of maize (GenBank accession number Z47554), also is expressed in postpollination maize endosperm (Elliott et al., 1996; Daniell et al., 1996).

**PhAp2A and the Petunia Class A Function: Isolation of PhAp2A Insertion Alleles**

In Arabidopsis, the class A gene Ap2 fulfills two roles in the process of floral organ identity determination: a cadastral function consisting of repression of the class C gene Agamous and an organ specification function in the perianth (sepal identity and determination of meristems in the second whorl).

To determine whether PhAp2A performs the same function in petunia that Ap2 does in Arabidopsis, we screened for dTph1 insertions in PhAp2A. Two of the five isolated PhAp2A insertion alleles had an insertion in the first exon of the gene. Surprisingly, W138 plants homozygous for these exon 1 insertions in PhAp2A do not exhibit a mutant phenotype in floral development.

**A Complete PhAp2A Knockout?**

Because attempts to obtain phenotypic phap2a mutants through cosuppression or antisense strategies have been equally unsuccessful (T. Maes, unpublished results), our first hypothesis is based on the assumption that the dTph1 insertions in exon 1 of PhAp2A knock out all of the potential functions of PHAP2A. We showed that the sequences of PhAp2B/PhAp2C, and their expression patterns during flower development, are very distinct from those of PhAp2A, making the hypothesis that they exert the same function improbable. Thus, the absence of a phenotype in the PhAp2A insertion mutants seems to contradict the hypothesis that homologs of the Ap2 gene of Arabidopsis play a crucial role as the class A function genes in the development of all angiosperm flowers.

Alternatively, the lack of a phenotype could be due to functional redundancy caused by the activity of another gene without any sequence homology with the Ap2 genes. This would imply that Arabidopsis would lack such a gene. Because the bl mutant shows analogous, albeit less pronounced, homeotic transformations reminiscent of A-type mutants of Arabidopsis, Bl is an obvious candidate for such a function. In Arabidopsis, at least two other mutants have been described that exhibit flowers displaying homeotic transformations similar to those in ap2 mutants: lug and clf (Liu and Meyerowitz, 1995; Goodrich et al., 1997). clf mutations have pleiotropic effects on flowering time, leaf shape, and flower morphology. In clf flowers, sepals and petals show partial transformation toward carpels and stamens. CLF seems to act later in flower development than AP2 and LUG (Liu and Meyerowitz, 1995; Goodrich et al., 1997). Another possibility is that the A function, as encoded by Ap2 of Arabidopsis, does not exist as such in petunia. The changes in the identity of perianth organs of A-type mutants found in petunia (bl) and in Antirrhinum species (fistulata and stylusa) can be attributed completely to the lack of cadastral activity, that is, to the lack of control of the expression domain of the C-type genes (Tsuchimoto et al., 1993; Motte et al., 1998). Until now, none of the A function mutants in these two species has been attributed to a mutation in an Ap2-like gene. In addition, Arabidopsis is the only species in which a gene has been identified that controls both sepal and petal specification and the expression of the class C gene Agamous. In Arabidopsis, Ap2 is required to establish the morphological features of the sepal epidermis, which differ from those of leaves in that the sepal epidermis does not have stellate trichomes (Bowman et al., 1989). In petunia, the inflorescence leaf epidermis and the sepal epidermis are indistinguishable, suggesting that the Ap2 ortholog of petunia has no function in the specification of sepal cell identity. If the only role of the PhAp2A gene in petunia consists of directly or indirectly negatively regulating the C function in the perianth, mutations that exclusively affect the transcription activation domain may not interfere with its function in flower development. This brings us to our second hypothesis.

**A Partial PhAp2A Knockout?**

Theoretically, truncated versions of the PHAP2A protein could be translated from the mRNA of the exon 1 insertion mutants we have obtained. In both cases, the truncated protein would be missing the putative transcription activation domain but would retain the putative nuclear localization signal and the AP2 domains. Such a truncated protein presumably still could function as a repressor and possibly regulate pMADS3 expression. Because PhAp2A overexpression can complement the ap2-1 mutation, the easiest
Figure 5. Insertion Mutagenesis of the PhAp2A Gene.

(A) Screening for insertion mutants. DNA pools of 1080 plants, organized in pools of 10 block samples, 12 row samples, and 9 column samples, were subjected to PCR using PhAp2A primer 002 in combination with the dTph1 inverted repeat primer. The PCR products were analyzed by DNA gel blot analysis using a PhAp2A-specific probe. Two positive plants were identified with the following coordinates: block 5, row 6, column 1 = V2025-6 and block 9, row 10, column 9 = V2047-10. The column signal of the second positive plant was missing in this screen but was identified in a similar experiment using PhAp2A primer 003. Marks indicate positive signals.

(B) Positions of the dTph1 insertions in PhAp2A and the positions of the primers used in the screening experiment. UTR, untranslated region.

(C) RT-PCR analysis of wild-type mRNA and PhAp2A(V2025-6) mRNA in the transposition-stabilized act /act background. Products were amplified from first strand cDNA by using PhAp2A primers 001 and 002. The wild-type product (WT; lane 1) is ~300 bp smaller than the product from the homozygous insertion mutant (INS; lane 2), which contains the dTph1 element (lane 3; PstI size marker).
way to verify whether we have obtained loss-of-function mutants, or if a truncated PhAp2A gene retains the ability to regulate Ag, would be to express a similarly truncated PhAp2A gene in the ap2-1 mutant of Arabidopsis.

**PhAp2A Behaves as a Functional A-Type Gene in Arabidopsis**

Promoter 35S-driven PhAp2A gene expression in Arabidopsis complements all mutant aspects of flower development of the ap2-1 mutation. Ectopic expression of Ag and the consequent homeotic changes in the perianth are inhibited, and the expression of genes required for the determination of sepal versus leaf identity is regulated correctly, in that normal sepals develop in the complemented flowers. In conclusion, the complementation of the ap2-1 mutant unequivocally proves our hypothesis that PhAp2A represents the functional ortholog of Ap2. Therefore, additional factors must be involved in the control of perianth identity in petunia. Although the general conservation of developmental control systems is widely accepted, there are examples in which divergence has occurred. A case in point seems to be the essential role played by *sonic hedgehog* (Shh) in the formation of body asymmetries in chick but not in mouse (Pagan-Westphal and Tabin, 1998). Here, we might have another example in which two orthologs have greatly diverged in at least some aspects of their function. Thus, either petunia PhAp2A has lost its perianth identity control function or Arabidopsis Ap2 has gained such a function. Further analyses involving a range of species are needed to clarify this point in more detail.

**METHODS**

**Plant Material**

*Petunia hybrida* line W138 contains a high copy number of the non-autonomous transposable element dTpH1, which requires the presence of an (active) Activator, located on chromosome 1, to undergo transposition (Gerats et al., 1990; Huits et al., 1995). Petunia lines W138 and W162 are homozygous for an unstable recessive mutation caused by an insertion of the nonautonomous element dTpH1 in the *An1* gene. *An1* controls the expression of several structural genes in the anthocyanin pathway, and *an1* mutants have a white corolla. The formation of red or pink revertant sectors and spots in line W138 results from excision of the dTpH1 element and can be used as a reporter system for transposition activity. Line W162 does not contain the Activator of the two-element system, and hence this line has white flowers (Huits et al., 1995). The *bl* mutant was in the R51 background.

**DNA Gel Blot Analysis**

DNA was extracted from petunia leaves as described by Souer et al. (1995). Twenty micrograms of DNA was digested and separated by agarose gel electrophoresis. Blotting and the composition of the hybridization buffer were as described by Amersham. α-32P-dCTP-labeled DNA probes were synthesized using the Megaprime DNA labeling kit (Amersham). Hybridizations and washes were performed at 60°C with 2 × SSC, 0.1% SDS and either 0.5 × SSC, 0.1% SDS (intermediate stringency) or 0.1 × SSC, 0.1% SDS (high stringency; 1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate).

**Isolation and Sequencing of the PhAp2 Genes**

The full-length Arabidopsis Ap2 cDNA was used to screen 105 plaque-forming units (PFUs) of a petunia λGEM4 cDNA library made from flower buds at low stringency. Initially, four positive clones were isolated that contained an 800-bp partial cDNA insert. This partial

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**Figure 6.** Complementation of the Arabidopsis ap2-1 Mutant by PhAp2A.

(A) Arabidopsis ap2-1 flower (grown at 26°C). The flower is from a transformant that did not exhibit complementation.

(B) Arabidopsis ap2-1::35S PhAp2A flower (grown at 26°C) exhibiting full complementation toward the wild type.

(C) Scanning electron micrograph of the seed coat of a noncomplementing ap2-1 transformant.

(D) Scanning electron micrograph of the seed coat of an ap2-1::35S PhAp2A transformant.

(E) Detail of (C).

(F) Detail of (D).

Bar in (C) and (D) = 100 μm; bar in (E) and (F) = 10 μm.
cDNA clone was used to screen 3 × 10^6 PPEs of a Uni-ZAP XR cDNA library from inflorescence meristems at medium stringency. The 15 positive clones that were isolated were analyzed by polymerase chain reaction (PCR) using two cPhAP2A-1–specific primers (p001 and p002). All clones corresponded to the same gene copy. Four full-length PhAP2A cDNA clones were isolated by screening 3 × 10^6 PPEs of a HybriZAP ovary cDNA library using a 300-bp PCR fragment amplified from a 1.1-kb PhAP2B genomic subclone as a probe.

The cPhAP2A-1 insert (a kind gift from Bart den Boer, Aventis Crop Sciences, Gent) was used to screen a V26 genomic library in λ at high stringency. Positive clones were analyzed by restriction analysis and represented the same gene. A 4.0-kb XbaI fragment cross-hybridizing with the cPhAP2-1 insert and a 3.2-kb flanking fragment were subcloned in pUC18, and deletion clones were generated using the method of Henikoff (1984). The PhAP2A gene contains nine introns; the positions of introns I to VI are conserved compared with those of the introns of the Arabidopsis AP2 gene.

To isolate PhAP2B and PhAP2C, we constructed a new genomic library from line W138 plants in λGem11 (Promega). The library was screened with the cPhAP2A-1 cDNA insert, and washes were at intermediate stringency (see DNA Gel Blot Analysis above). Twenty-nine clones were isolated in the primary screen, 15 of which were analyzed further. They could be divided based on their restriction patterns into three groups that corresponded to the three gene copies observed in DNA gel blots. A 1.2-kb SstI-XbaI PhAP2B fragment and a 1.1-kb EcoRI-HindIII PhAP2C fragment were subcloned and sequenced.

Sequences were determined by the dideoxy chain termination method (Sanger et al., 1977) using fluorescent dye terminators and an ABI1373A or an ABI1377 automatic sequencer. Gaps in the sequence were closed using a primer-based strategy.

Chromosomal Localization of PhAP2A, PhAP2B, and PhAP2C

We detected HindIII restriction fragment length polymorphisms (RFLPs) for the three PhAP2c copies between lines V26 and W137. To determine the map positions of PhAP2A, PhAP2B, and PhAP2C, we crossed line V26 to line W137, and the F1 hybrid was backcrossed to line W137. A population of 61 individuals was used to construct a genetic map. Linkage between 183 amplified fragment length polymorphism (AFLP) markers, obtained from 14 different primer combinations (EcoRI plus three selective nucleotides, Msel plus three selective nucleotides), four phenotypic markers (H1 on chromosome 1, F1 on chromosome 2, An11 on chromosome 3, and Rf on chromosome 6), and the PhAP2 RFLPs in this backcross population, was analyzed by the JoinMap program (Stam, 1993). The AFLP linkage groups identified in the progeny of the (V26 × W137) × W137 test cross could be anchored to their specific chromosomes by linkage to the phenotypic markers and by comparison with the locations of 40 AFLP markers that had been located previously in a different set of crosses (Gerats et al., 1995).

The linkage between PhAP2A and the Bl locus, both on chromosome 4, was investigated further using a mutant PhAP2A allele as a segregating marker. Plant T2194-14 is heterozygous for a transposon insertion in intron VIII (see Transposon Insertion Mutagenesis of PhAP2A). T2194-14 was crossed to line Rs1 (bl/bl), and F1 hybrids that contained the PhAP2A insertion allele were selfed. All of the F1 plants were wild type. Of 96 F2 plants, 26 exhibited a bl phenotype. PCR analysis, using gene-specific primers flanking the transposon insertion, showed that seven of 20 bl F2 plants tested carried the PhAP2A(T2194-14) allele. Because PhAP2A(T2194-14) homozygotes are wild type, and bl segregated as a normal recessive mutation, and because seven of 20 bl mutants contain the insertion allele, we conclude that PhAP2A does not coincide with Bl. Moreover, bl mutants that are homozygous for the PhAP2A(T2194-14) insertion allele exhibit a phenotype similar to that of bl mutants that carry wild-type PhAP2A alleles.

In Situ Hybridization Studies

Petunia tissue (line W138) was fixed in 3.7% formaldehyde, 5% acetone, and 50% ethanol, cleared in Histoclear (National Diagnostics, Atlanta, GA), and embedded in paraffin. Consecutive 10-μm tissue sections were placed on sets of dimethylsilane-treated (Sigma) microscope slides so that comparable sections could be hybridized with different probes. The cDNA inserts of cPhAP2A-2 and pMADS3 were amplified from the plasmid using the T3 and T7 sequencing primers. PhAP2A and pMADS3 antisense mRNA and sense mRNA (control) probes were 32P-labeled mRNA probes synthesized from this PCR fragment by using the T7 and T3 RNA polymerase (Promega). An ~300-bp fragment (nucleotides 198 to 596 of the PhAP2B cDNA sequence shown in Figure 3) and a 300-bp fragment were amplified from genomic subclones of PhAP2B and PhAP2C by using the T7 primer and a degenerate, chimeric T3-exon 2 PhAP2 primer (5′-AATAACCCCTCATAAAGGAATATGTGATGAT-3′). The presence of a transcriptional start site was confirmed by RT-PCR analysis using a set of two gene-specific primers, p001 (5′-GCCTAAAGCGATCGATTTTTCGAC-3′) and p010 (5′-CGGATGCTGCGACATGATGCGTCATGCGGC-3′) and by RT-PCR analysis using a set of two gene-specific primers, p001 (5′-GTGGGGCTGCTGATGCGTCATGCGGC-3′) and p010 (5′-CGATGCTGACATGATGCGTCATGCGGC-3′) were in combination with p002 to analyze the progyn of the primary V2025-6 and V2116-10 mutants, respectively. The PCRs were performed in 50 μL of 1 × PCR buffer (Perkin-Elmer) supplied with MgCl2 (1.5 mM) and 1 unit of Taq polymerase (Perkin-Elmer) and consisted of 35 cycles of 30 sec denaturing at 94°C, 30 sec annealing at 55°C, and 1.5 min polymerization at 72°C. DNA fragments flanking the dTph1 elements in PhAP2A were amplified by PCR, cloned in pGEM-T, and sequenced to determine their exact insertion positions.

Reverse Transcription–PCR

Total RNA from flower buds was treated with DNase, extracted with phenol/chloroform, and precipitated, and 1 μg was reverse tran-
scribed from an oligo-dT primer by using the Superscript premi- amplification system for first-strand synthesis (Gibco BRL). The first-strand cDNA was used as a template for PCR amplification with either p001/p002 (V2025-6) or p010/p002 (V2116-10).

Overexpression of PhAp2A in Arabidopsis thaliana

Primers naste22 (5'-GAATTCCTAATAGCTTTTCAAAATGTC-3') and naste23 (5'-CGAATTCGCTAGAGGTGTCGATAAGA-3') were used to amplify the PhAp2A coding sequences. The fragment obtained was cloned in pGEM-T (Promega), and the sequence was verified. An ~1600-bp PhAp2A fragment was cut from this plasmid by using the BglII site in primer naste22 and the HincII site from the polylinker of pGEM-T. The binary vector pBIN m-gfp5-ER (kindly provided by James Haseloff, Cambridge, UK) was digested with SacI, which provided the gfp5-ER (kindly provided by James Haseloff, Cambridge, UK) was digested with SacI, which provided the gfp5-ER site in the vector pBIN m-gfp5-ER. The resulting overexpression vector pBIN19-35S-PhAp2A was introduced into Agrobacterium tumefaciens C58C1(Rif) (pMP90[Gen] by triparental mating. The Arabidopsis ap2-2 mutant (kindly provided by Maarten Koornneef, LUW, Wageningen) was transformed as described by Clarke et al. (1992). Kanamycin-resistant calli and shoots were selected at 23 °C, and the shoots and selfed progeny of primary transformants were grown at 26 °C under long-day conditions (16 hr light and 8 hr dark).

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