The *Arabidopsis* floral homeotic gene *APETALA3* differentially regulates intercellular signaling required for petal and stamen development

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SUMMARY

Cell-cell signaling is crucial for the coordination of cell division and differentiation during plant organogenesis. We have developed a novel mosaic analysis method for *Arabidopsis*, based on the maize Ac/Ds transposable element system, to assess the requirements of individual genes in intercellular signaling. Using this strategy, we have shown that the floral homeotic *APETALA3* (*AP3*) gene has distinct roles in regulating intercellular signaling in different tissues. In petals, *AP3* acts primarily in a cell-autonomous fashion to regulate cell type differentiation, but its function is also required in a non-cell-autonomous fashion to regulate organ shape. In contrast, *AP3*-regulated intercellular interactions are required for conferring both cell type identity and organ shape and size in the stamens. Using antibodies raised against *AP3*, we have shown that the *AP3* protein does not traffic between cells. These observations imply that *AP3* acts by differentially regulating the production of intercellular signals in a whorl-specific manner.

Key words: *APETALA3*, *Arabidopsis thaliana*, Homeotic gene, Non-autonomy, Flower development

INTRODUCTION

Flowers are particularly amenable to the genetic analysis of organ identity, due to the existence of single locus homeotic mutations that transform the identity of the floral organs. The flower of *Arabidopsis* consists of four whorls of organs: sepals, petals, stamens and carpels (Fig. 1B) which arise by the proliferation of cells derived from the shoot apical meristem. Studies in *Arabidopsis* and other species have demonstrated that flower cell fates are dependent on position, not lineage (Jenik and Irish, 2000; Stewart and Burk, 1970). The floral organ identity genes are good candidates to be involved in regulating the positional signaling required for floral organogenesis. In *Arabidopsis*, two floral organ identity genes, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) are required to specify petal and stamen identity. Flowers homozygous for strong alleles, like *ap3-3* or *pi-1*, display homeotic transformations of petals into sepals and stamens into carpeloid organs (Goto and Meyerowitz, 1994; Jack et al., 1992) (Fig. 1C). *AP3* and *PI* are expressed throughout petals and stamens during organ development (Goto and Meyerowitz, 1994; Jack et al., 1992), and the continuous expression of *AP3* is required to specify organ identity (Bowman et al., 1989).

Both *AP3* and *PI* encode products that contain a MADS-domain, a DNA-binding motif that has been identified in a number of yeast, plant and mammalian transcription factors, and so are likely to act by transcriptionally regulating a suite of downstream genes (Goto and Meyerowitz, 1994; Jack et al., 1992). The *AP3* and *PI* gene products form a heterodimer, and their dimerization is necessary for DNA binding and for nuclear localization (McGonigle et al., 1996; Riechmann et al., 1996). While few downstream targets have been identified, *AP3* expression is required for the autoregulatory maintenance of *AP3* transcription after stage 5 of floral development (Jack et al., 1994; for stages of development see Smyth et al., 1990). Similarly, *AP3* expression is required for the maintenance of *PI* transcription after stage 5 (Goto and Meyerowitz, 1994). These regulatory interactions may be direct, since *AP3/PI* heterodimers have been shown to bind to regulatory sequences in the *AP3* promoter (Hill et al., 1998; Tilly et al., 1998). Despite their constant and ubiquitous expression during petal and stamen development, the mechanisms by which *AP3* and/or *PI* function to specify particular petal and stamen tissue types has not yet been ascertained.

One way to study the mode of action of a gene is by using mosaic analysis to generate organs or tissues that contain cells of different genotypes. In plants, graft chimeras have been used to address the role of cell-cell signaling in organogenesis, but such techniques are difficult to implement in *Arabidopsis* due to its small size (reviewed by Szymkowiak, 1996). Instead, such issues have begun to be addressed in *Arabidopsis* by using revertible alleles, X-rays and FLP or cre recombinases to generate genetic mosaics (Bouhidel and Irish, 1996; Furner et al., 1996; Helariutta et al., 2000; Sessions et al., 2000; Sieburth et al., 1998). Previous mosaic analyses of the floral homeotic genes *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*) in *Antirrhinum majus* (Perbal et al., 1996), and *APETALA1* (*AP1*) and *AGAMOUS* (*AG*) in *Arabidopsis* (Sessions et al., 2000;
Sieburth et al., 1998) have shown that these genes have both cell-autonomous and non-cell-autonomous effects on organ morphogenesis.

We have developed a new strategy, based on the heterologous maize Activator/Dissociation (Ac/Ds) transposable element system, to reproducibly and reliably generate mutant or wild-type sectors in Arabidopsis. The shoot and inflorescence apical meristems (SAM and IM, respectively), which give rise to all the aerial portions of the plant, are composed of three layers of cells, termed L1, L2 and L3. Mosaic analysis takes advantage of these clonal layers: by altering the genotype of a meristematic cell it is possible to generate flowers in which a whole cell layer is genetically different from the others. Using the Ac/Ds system we generated flowers mosaic for AP3. We have found that AP3 has both autonomous and non-autonomous effects during organ development, pointing to a role in cell-cell signaling. Our analyses further demonstrate that AP3 can regulate cell fate autonomously, while it acts non-autonomously to control overall organ shape and size. Surprisingly, these effects are different depending on the organ or the layer involved, suggesting that AP3 may utilize different mechanisms to regulate petal versus stamen development.

It has been postulated that, in plants, transcriptional regulators could exert their non-autonomous effects by trafficking between cells (Mezitt and Lucas, 1996). This hypothesis was based on studies in maize SAMs, which showed that while the mRNA for the homeobox KNOTTED1 (KN1) could only be detected in the internal cell layers, KN1 protein was also present in the epidermis (Jackson et al., 1994). Similar observations were also made in Antirrhinum flowers mosaic for the DEF or GLO floral homeotic genes (Perbal et al., 1996), and in Arabidopsis flowers expressing the floral meristem identity gene LEAFY (LFY) in the L1 only (Sessions et al., 2000). We tested this hypothesis by examining the cellular localization of AP3 protein in mosaic flowers. Our results indicate that the non-autonomous effects of AP3 are not due to AP3 protein movement, but to the action of as yet unidentified downstream genes. Furthermore, the distribution of the PI protein coincided with that of the AP3 protein, suggesting that AP3 regulates PI expression cell-autonomously. Comparisons of our results for AP3 and those obtained for its Antirrhinum ortholog DEF suggest that the mechanisms by which these Arabidopsis and Antirrhinum floral homeotic genes act to specify organ identity have diverged during the evolution of these species.

MATERIALS AND METHODS

Cloning and generation of transgenic plant lines

To assemble 35S::Ap3G (Fig. 1A), a fragment from pBl221 (Clontech, Palo Alto, CA) that contained the 35S promoter, GUS and a nos terminator was cloned into the plant transformation vector pPZZP221 (Hajdukiewicz et al., 1994), to generate p235G. The AP3 minimal promoter (+1 to –78) was amplified by PCR and fused to the AP3 cDNA. The AP3 minimal promoter-cDNA fragment was cloned into the unique BamHI site of the SnaBI-Ds73 element (a 660 bp Ds element kindly provided by Steve Dellaporta, Yale University). The DsAP3 element was then inserted into p235G, between the 35S promoter and GUS, to generate p35SpA3G.

For AP3pGA3 (Fig. 1A), a nos terminator was first inserted downstream of a promoterless AP3 cDNA. A SnaBI-Ds73 element containing the GUS coding region was then cloned upstream of the AP3 cDNA. pAP3pGA3 was assembled in a three way ligation involving a 927 bp AP3 promoter (D3 fragment; Hill et al., 1998), the DsGUS-AP3-nos and pPZP221.

Both constructs were transformed into the Landsberg erecta (Ler) ecotype. Three independent transgenic lines for each construct were used for the mosaic studies. All the lines used contained a single copy of the transgene (determined by the segregation of gentamycin resistance and by Southern hybridization, data not shown). All the lines containing the same construct showed the same phenotype.

Generation of mosaic plants

The 35S::Ac transgenic line, containing an Ac transposase driven by a 35S promoter (Sundaresan et al., 1995), was transformed into the Landsberg erecta ecotype. T2 generation 35SpA3G or AP3pGA3 transgenic plants were also crossed into the ap3-3 background. To generate mosaic plants we performed the following crosses: 35SpA3G/35SpA3G; ap3-3/ap3-3 × 35S::Ac/35S::Ac: ap3-3/ap3-3 or AP3pGA3/35SpA3G; ap3-3/ap3-3 × 35S::Ac/35S::Ac: ap3-3/ap3-3. F1 plants were screened for sectors as described in the Results. Plants were grown as described previously (Jenik and Irish, 2000). Transgenic plants were selected on 50 μg/ml kanamycin and/or 100 μg/ml gentamycin. The genotyping for ap3-3, when necessary, was carried out by using allele-specific PCR (PCR primers and amplification conditions are available on request). The AP3-342 and AP3K2 primer pair (Fig. 1A) was used to detect excisions in plants carrying AP3pGA3.

Production of antibodies

To generate polyclonal antisera against the AP3 and PI proteins, a fragment spanning positions 239 to the end of the AP3 cDNA (amino acid 68 to the end) was used. For PI, a fragment spanning positions 218 to the end of the cDNA (amino acid 67 to the end) was used. Neither fragment contained the MADS-box. The DNA fragments were subcloned into pET-15b (Novagen, Madison, WI), so that the fragments spanning positions 239 to the end of the cDNA (amino acid 68 to the end) was used. For AP3, a fragment spanning positions 3 to the end of the cDNA (amino acid 18 to the end) was used. Neither fragment contained the MADS-box. The DNA fragments were subcloned into pET-15b (Novagen, Madison, WI), so that the fragments were in frame with the N-terminal His-tag. The fusion proteins were expressed in E. coli BL21 and purified (after resuspension in 6 M urea) with His-Bind resin (Novagen), following the manufacturer’s instructions. The purified proteins were injected into rats (for His-PI) or rabbits (for His-AP3).

GST fusion proteins were also generated by cloning similar fragments into pGEX-5X-2 (Pharmacia Biotech, Uppsala, Sweden). The GST fusion proteins were expressed in E. coli BL21 and resuspended in 6 M urea. After dialysis, the proteins were purified with glutathione-Sepharose 4B beads (Pharmacia Biotech). GST-AP3 or GST-PI were covalently bound to HiTrap affinity columns (Pharmacia Biotech), which were used to affinity-purify the corresponding antisera. The affinity purified anti-AP3 did not recognize PI on a western blot, and vice versa. Both antibodies revealed the expected wild-type expression patterns when used to probe tissue sections of Ler inflorescences. Neither antibody showed any signal when used to probe tissue sections of the corresponding mutant (ap3-3: stop at codon 18, or pi-1: stop at codon 80) (data not shown).

Histochemistry, immunohistochemistry, and microscopy

The protocols for the detection of GUS activity and for immunolocalizations have been described previously (Jenik and Irish, 2000). For immunolocalizations in this study, 0.02% saponin was used instead of 0.1% Tween 20. The dilutions of the purified antibodies were as follows: anti-AP3 1:500, anti-PI 1:100, anti-GUS (Molecular Probes, Eugene, OR) 1:1000, secondary antibodies (alkaline phosphatase-conjugated goat anti-rat or anti-rabbit, Jackson ImmunoResearch, West Grove, PA) 1:2000. The results were analyzed with a Zeiss Axioshot microscope.
shown to function in maize is precise, leaving an 8 bp footprint (Fedoroff, 1989). The Ac/Ds heterologous maize respectively). In order to produce such mosaics, we utilized the from the other layers (periclinal or mericlinal inflorescences, early in development will result in inflorescences that contain Genetically marking a single cell in one of the meristem layers RESULTS

poor (Fig. 1D), presumably due to the relatively weak levels of the 35S::Ac transposase we used a -containing constructs. For a constitutive source of generated transgenic lines containing single copy inserts of one transposase gene is driven by the Cauliflower Mosaic Virus promoter driving an promoter (Sundaresan et al., 1995). For both types of constructs, we selected early excision events, which could not be used to screen for sectors in plants containing this element.

The second construct, AP3pGA3 (Fig. 1A), was used to generate wild-type sectors in an ap3-3 background. This construct contains a 927 bp fragment of the AP3 promoter that is sufficient to confer a wild-type expression pattern (Hill et al., 1998). In AP3pGA3, this promoter drives the expression of GUS placed between the ends of a Ds element, followed by the AP3 cDNA. Standard genetic crosses were used to produce AP3pGA3/+; 35S::Ac/+; ap3-3/3/ap3-3 plants. Germinal excisions (excision events present in the gametes and transmitted to the next generation, see below) show that excision of the DsGUS element reconstituted AP3 expression and was sufficient to fully rescue the ap3-3 mutant phenotype (Fig. 1B). To identify mosaic plants we screened AP3pGA3/+; 35S::Ac/+; ap3-3/3/ap3-3 plants for changes in the ap3-3 mutant background and could not be used to screen for sectors in plants containing this construct.

In this study, the genotypes of the layers in the sectored flowers are designated as ‘L1/L2/L3’. For instance, in an AP3/3/3 mosaic flower all cells derived from the L1 and L2 are genetically wild type (AP3) and all cells derived from the L3 are genetically ap3-3. The results of the analyses are summarized in Table 1.

Table 1. Summary of the analysis of mosaic second and third whorls

<table>
<thead>
<tr>
<th>Layer genotypes (L1/L2/L3)</th>
<th>Transgenic background</th>
<th>Second whorl phenotype</th>
<th>Third whorl phenotype</th>
<th>Number of inflorescences analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP3/3/3/3/3/3</td>
<td>Ler control</td>
<td>Petals</td>
<td>Stamens</td>
<td>20</td>
</tr>
<tr>
<td>ap3/ap3/ap3</td>
<td>ap3-3 control</td>
<td>Sepals</td>
<td>Carpeloid</td>
<td>20</td>
</tr>
<tr>
<td>AP3/ap3/ap3</td>
<td>AP3pGA3</td>
<td>Green mesophyll, petaloid epidermis and shape</td>
<td>Carpeloid</td>
<td>20</td>
</tr>
<tr>
<td>35SpA3G</td>
<td>n.d.</td>
<td>AP3pGA3</td>
<td>Carpeloid</td>
<td>1</td>
</tr>
<tr>
<td>35SpA3G</td>
<td>n.d.</td>
<td>AP3pGA3</td>
<td>Carpeloid</td>
<td>19</td>
</tr>
<tr>
<td>35SpA3G</td>
<td>n.d.</td>
<td>AP3pGA3</td>
<td>Carpeloid</td>
<td>35</td>
</tr>
<tr>
<td>35SpA3G</td>
<td>n.d.</td>
<td>AP3pGA3</td>
<td>Carpeloid</td>
<td>67</td>
</tr>
<tr>
<td>35SpA3G</td>
<td>n.d.</td>
<td>AP3pGA3</td>
<td>Staminoid organs</td>
<td>1</td>
</tr>
<tr>
<td>35SpA3G</td>
<td>n.d.</td>
<td>AP3pGA3</td>
<td>Staminoid organs</td>
<td>31</td>
</tr>
<tr>
<td>35SpA3G</td>
<td>n.d.</td>
<td>AP3pGA3</td>
<td>Stamen identity with almost no carpel characteristics.</td>
<td>17</td>
</tr>
</tbody>
</table>
| 35SpA3G                   | n.d.                  | AP3pGA3               | Shape not normal     | Total=279

§ n.d.: not determined. *At least one inflorescence of this type, detected by PCR (see text).

For scanning electron microscopy (SEM) we followed previously established protocols (Irish and Sussex, 1990). Images were processed using Adobe Photoshop (Adobe Systems, Mountain View, CA).

Generation of mosaics

Genetically marking a single cell in one of the meristem layers early in development will result in inflorescences that contain a whole layer, or a part of a layer, that is genotypically different from the other layers (periclinal or mericlinal inflorescences, respectively). In order to produce such mosaics, we utilized the heterologous maize Ac/Ds transposable element system. Ds is a non-autonomous transposable element and requires Ac transposase supplied in the epidermis and shape expression conferred by the 35S promoter in the second whorl (Jack et al., 1994; Jenik and Irish, 2000). Standard genetic crosses were used to generate 35SpA3G/+; 35S::Ac/+; ap3-3/3/ap3-3 plants, which were screened for excision events by GUS staining. Because the 35SpA3G transgene shows poor rescue of petals, we only analyzed third whorl structures in these mosaic plants.

The second construct, AP3pGA3 (Fig. 1A), was used to generate wild-type sectors in an ap3-3 background. This construct contains a 927 bp fragment of the AP3 promoter (AP3p) that is sufficient to confer a wild-type expression pattern (Hill et al., 1998). In AP3pGA3, this promoter drives the expression of GUS placed between the ends of a Ds element, followed by the AP3 cDNA. Standard genetic crosses were used to produce AP3pGA3/+; 35S::Ac/+; ap3-3/3/ap3-3 plants. Germinal excisions (excision events present in the gametes and transmitted to the next generation, see below) show that excision of the DsGUS element reconstituted AP3 expression and was sufficient to fully rescue the ap3-3 mutant phenotype (Fig. 1B). To identify mosaic plants we screened AP3pGA3/+; 35S::Ac/+; ap3-3/3/ap3-3 plants for changes in the ap3-3 mutant background. Sectors that do not show an ap3-3 mutant phenotype would not be identified with this strategy. Since the AP3 gene autoregulates (Jack et al., 1994), GUS is not expressed in the ap3-3 mutant background and could not be used to screen for sectors in plants containing this construct.

In this study, the genotypes of the layers in the sectored flowers are designated as ‘L1/L2/L3’. For instance, in an AP3/3/3 mosaic flower all cells derived from the L1 and L2 are genetically wild type (AP3) and all cells derived from the L3 are genetically ap3-3. The results of the analyses are summarized in Table 1.

Analysis of mosaic second whorl organs

We screened 544 AP3pGA3/+; 35S::Ac/+; ap3-3/3/ap3-3
inflorescences (98 plants) for wild-type sectors. We found 44 inflorescences (15 plants) in which the flowers appeared different from ap3-3 flowers. There were two distinct classes of floral phenotypes: the first class comprised 20 inflorescences, the second 24 inflorescences (Fig. 2).

In the first class of flowers we examined tissue sections of flowers from 9 inflorescences using an anti-AP3 antibody to determine the genotype of the cell layers. In these flowers, the AP3 protein was present only in the epidermis of the second whorl organs, which is derived from the L1 (Fig. 2C). We never observed AP3 protein in L2-derived cells (mesophyll) in these mosaics. This L1-specific expression pattern was also observed in early floral stages (Fig. 2E). These observations indicated that these flowers were AP3/ap3/ap3 mosaics. The second whorl AP3/ap3/ap3 mosaic organs were also somewhat larger than

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**Fig. 1.** Experimental strategy. (A) Schematic representation of the constructs used in this study, before and after the precise excision of the Ds element. 5' and 3' indicate the 5' (170 bp) and 3' (490 bp) moieties of the Ds element. The primers used to test for excisions are indicated (AP3-342, AP3K2). (B) ap3-3 flower carrying a germinal excision of AP3pGA3. The excised derivative restores the wild-type phenotype. (C) ap3-3 flower; the first whorl sepals have been removed to show the second whorl sepaloid organs (arrows). (D) 35SpA3G/+; ap3-3/ap3-3 flower has normal stamens (arrowhead) but the petals are poorly rescued (arrow). (E-G) Consecutive longitudinal sections of a 35SpA3G/+; ap3-3/ap3-3 mosaic flower (stage 3). (E) The L1 and L2 are genotypically wild type and the AP3 protein is present. (F) The L3 is genotypically ap3-3 and expresses GUS protein. (G) PI protein is present in all layers until stage 5, but at higher levels in the layers containing AP3 protein. Scale bar, 10 μm (E-G).

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**Fig. 2.** Analysis of mosaic second whorl organs. (A) Ler flower, with the sepals removed, showing the typical shape of the petals. (B) Mature AP3/ap3/ap3 flower. The second whorl organs (arrow) are green and small, but petaloid in shape (one sepal has been removed). (C-F,I-L) Sections of mosaic flowers probed with either anti-AP3 or anti-PI. Sections in C,D,I are transverse, sections in E,F,J,K,L are longitudinal. Whorls are numbered. (C,D) Mature AP3/ap3/ap3 flowers. Both AP3 (C) and PI (D) proteins are present only in the epidermis of the second whorl organs, which is derived from the L1 (Fig. 2C). We never observed AP3 protein in L2-derived cells (mesophyll) in these mosaics. This L1-specific expression pattern was also observed in early floral stages (Fig. 2E). These observations indicated that these flowers were AP3/ap3/ap3 mosaics. The second whorl organs of AP3/ap3/ap3 flowers were green like sepals but they had a rounded distal portion and a narrow proximal portion, in a shape that is similar to that of normal petals. In contrast, sepals have a strap-like shape with a jagged tip. The second whorl AP3/ap3/ap3 mosaic organs were also somewhat larger than...
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The epidermis of AP3/ap3/ap3 second whorl organs resembles that of wild-type petals (WT), while the epidermis of ap3/AP3/ap3 second whorl organs appears sepaloid, as in the mutant (ap3-3). Scale bar, 10 μm.

Fig. 3. Epidermal characteristics of mosaic second whorl organs. SEM micrographs of the abaxial and adaxial epidermis of the distal portion of second whorl organs in the different mosaic types. The epidermis of AP3/ap3/ap3 second whorl organs resembles that of wild-type petals (WT), while the epidermis of ap3/AP3/ap3 second whorl organs appears sepaloid, as in the mutant (ap3-3). Scale bar, 10 μm.

The mesophyll were, in contrast, sepaloid in nature, as indicated by the presence of chloroplasts (Fig. 2B).

To determine the genotype of the cell layers of the second class of flowers, we examined AP3 protein distribution in 10 inflorescences. AP3 protein was detected only in the mesophyll of the second whorl organs (derived from the L2) and not in the epidermis of this class of flowers (Fig. 2I). L2-restricted expression could also be clearly seen in early floral stages (Fig. 2K). We concluded that these flowers were ap3/AP3/ap3 mosaics. In ap3/AP3/ap3 flowers the second whorl organs were white and larger than sepals, but had a shape resembling that of a sepal, not of a petal (Fig. 2G,H). Examination of the second whorl epidermal cells using SEM showed that these cells appeared identical to sepal epidermal cells (Fig. 3). The mesophyll cells, in contrast, had petal characteristics, in that they lacked chloroplasts (Fig. 2G,H).

We obtained an equivalent number of AP3/ap3/ap3 and ap3/AP3/ap3 mosaic inflorescences. We expected to obtain a similar number of ap3/ap3/AP3 mosaic inflorescences, which may have had a phenotype distinct from ap3-3. However, we did not obtain a third class of phenotype indicative of the presence of ap3/ap3/AP3 mosaics. To test for the presence of undetected excision events in the L3, we carried out a PCR analysis on individual AP3pGA3/+; 35S::Ac/+, ap3-3/ap3-3 inflorescences that had a typical ap3-3 mutant phenotype (Fig. 1A). Within this population we found a frequency of excisions (3%) that was consistent with the number of ap3/ap3/AP3 sectors expected (data not shown). These results suggest that ap3/ap3/AP3 sectors were present in our sample, but that the expression of AP3 in the L3 alone does not have a phenotypic effect in the second or third whorls. The L3 does contribute to the vascular cylinder of the stamens, but it does not contribute to the petals and has little or no contribution to the sepals (Jenik and Irish, 2000).

Fig. 4. Analysis of mosaic third whorl organs. (A-C) Mature ap3/AP3/AP3 flowers. (A) GUS-stained flower. The stamens are partially rescued (arrow) and contain locules with pollen grains, but the size and shape of these organs are not normal. (B,C) Longitudinal sections of a flower showing the presence of AP3 protein in the internal tissues of the partially rescued stamen (B), and of GUS protein in the epidermis (C). (D-G) Mature AP3/ap3/AP3 flowers. The flowers show no rescue of the stamens. (D) GUS-stained flower. Longitudinal sections of such an AP3/ap3/AP3 flower show expression of AP3 (E) and PI (G) proteins in derivatives of the L1 and L3 in the second whorl (but not in the epidermis of the third whorl filament, arrowhead). In these flowers, GUS protein is expressed in derivatives of the L2 (F). (H-K) Mature AP3/AP3/ap3 flowers. (H) GUS-stained flowers. The flowers have apparently normal stamens (arrow; sepals and petals removed). Longitudinal sections of an AP3/AP3/ap3 flower showing the complementary patterns of expression in the stamen of the AP3 protein (I) and the GUS protein (J). (K) Section of a GUS-stained mericlinal flower (GUS staining appears pink under dark-field illumination). The right side of the flower is genotypically AP3/ap3/ap3 and has filaments instead of stamens (arrowheads), while the left side is AP3/AP3/ap3 and has normal looking stamens (arrows). The mericlinal sector boundary is indicated by a dotted line. Whorls are numbered. Scale bar, 50 μm (B,C,E-G), 80 μm (I,J), 160 μm (K). In B,E,I, whorls are numbered.
We also examined the distribution of PI protein in both AP3/ap3/ap3 and ap3/AP3/ap3 mosaic flowers using an anti-PI antibody. In all the sections analyzed, the PI protein always localized to the same cells as the AP3 protein (Fig. 2C,D,I,J), similar to what is observed in wild-type flowers (P. D. J. and V. F. I., unpublished results). The only exception was in stage-3 to -5 flowers, where the PI protein is observed in all layers (Fig. 2FL). This is consistent with previous results that show that the expression of AP3 is necessary for the maintenance of AP3 and PI expression only after stage 5 (Goto and Meyerowitz, 1994; Jack et al., 1992). These results indicate that AP3 regulates PI expression cell autonomously after stage 5.

Our results show that AP3 has both cell autonomous and non-autonomous effects during petal development (Fig. 7). The AP3 gene specifies cellular identity autonomously: the cellular phenotype and genotype coincide. AP3 cells show petal-like features (petaloid epidermal cell characteristics in AP3/ap3/ap3 organs, mesophyll lacking chloroplasts in ap3/AP3/ap3 organs), while ap3-3 cells have sepal-like traits (mesophyll containing chloroplasts in AP3/ap3/ap3 organs, sepaloid epidermal cells in ap3/AP3/ap3 organs). However, AP3 also has apparent non-autonomous effects. The genotype of the L1 has a strong influence on organ shape, while that of the L2 has an effect on organ size. Together these results suggest that, in the second whorl, AP3 acts cell autonomously in the control of cell type identity, but has non-autonomous effects on the regulation of organ morphogenesis. We observed no movement of the AP3 protein between cell layers. This implies that, while AP3 is required for cell-cell signaling events responsible for controlling petal shape and size, the signal is not the AP3 protein.

**Analysis of mosaic third whorl organs**

Third whorl phenotypes were classified into three classes. Flowers showed ‘no rescue’ when the stamens were absent, transformed into filaments or presented obvious carpeloid characteristics (flat or tubular organs with ovules and stigmatic tissue, no visible locules with pollen grains). ‘Partially rescued’ organs had no apparent carpeloid traits and contained yellow locules with pollen grains, although the shape of the organs did not correspond to that of a wild-type stamen. ‘Rescued’ organs were those that looked like, or almost like, wild-type stamens and produced normal pollen grains.

The AP3/ap3/ap3 and ap3/AP3/ap3 mosaic flowers (identified by the second whorl phenotype) and the putative ap3/ap3/AP3 mosaic flowers (identified by PCR), derived from the AP3pGA3 construct, did not produce stamens (Fig. 2A-D,G-I), even though the fragment of the AP3 promoter driving the AP3 cDNA was capable of fully rescuing the ap3-3 mutant phenotype when expressed in all layers (Fig. 1B). This indicates that the expression of AP3 in the L1, L2 or L3 alone is not sufficient to confer stamen identity to the third whorl (Table 1).

To obtain a wider variety of sector types, we screened 35SpAP3G/+; 35S:Ac/+; ap3-3/ap3-3 plants by staining inflorescences for GUS activity and selecting those that showed large periclinal or mericlinal ap3-3 (GUS positive) sectors. The layer composition of these mosaics could be determined in whole mounts of GUS stained flowers (Jenik and Irish, 2000). The rescue of the stamen phenotype by 35SpAP3G was not always complete, due to variable levels of expression from the 35S promoter in the flower (Jack et al., 1994; Jenik and Irish, 2000). We therefore only used plants in which the non-sectored control inflorescences on the same plant showed fully rescued stamens. We screened 371 inflorescences from 116 plants. We obtained 195 sectors from 174 inflorescences (a few inflorescences contained more than one type of sector). The types of sectors and their phenotypes are summarized in Table 1. Some GUS-stained inflorescences were sectioned and analyzed using light microscopy. We also examined 29 inflorescences with anti-AP3, anti-PI and anti-GUS antibodies (8 AP3/ap3/ap3, 1 ap3/ap3/AP3, 6 ap3/AP3/ap3, 8 AP3/ap3/AP3 and 6 AP3/AP3/ap3 mosaics). Results were consistent for each mosaic type.

We found three different classes of third whorl phenotypes (Table 1). The first class of phenotype was found in ap3/AP3/ap3 mosaic flowers. In these mosaics we obtained a partial rescue of the stamens. The partially rescued stamens were shorter and usually flatter than wild-type organs, with less distinct filaments and anthers (Figs 4A and 5A,B,D,E). They generally contained two locules with morphologically normal pollen grains. In these partially rescued stamens, AP3 (and PI) proteins were present in the L2 and L3 but not in the epidermis (Fig. 4B,C). We examined these organs using SEM and found that the epidermal cells were staminoid in shape and size, and clearly different from carpel epidermal cells (Fig. 5C,F,I). However, the epidermis at the edge of the organ still had carpeloid characteristics, as indicated by the presence of short stigmatic papillae and small, aborted ovules (Fig. 5D,E). These results indicate that the expression of AP3 in the L1 is required for normal morphogenesis and growth of the third whorl organs. However, AP3 function in L1 cells is not necessary to specify stamen-specific epidermal cell types. These observations indicate that expression of AP3 in the L2 and L3 is sufficient to convey information pertaining to cell type specification to the L1-derived epidermis, in a non-autonomous fashion.

The second class of phenotype included all the flowers in which cells derived from the L2 were mutant (AP3/ap3/ap3, ap3/ap3/AP3 and AP3/ap3/AP3 mosaics; Fig. 4D-G). These flowers showed no rescue of the third whorl to stamens, indicating that the expression of AP3 in the L2 is necessary (but not sufficient, see above) to confer stamen identity.

The third class of phenotype was observed in AP3/AP3/ap3 mosaic flowers, which had normal stamens (Fig. 4H,K). Again in this class, the presence of the AP3 and PI proteins was restricted to the cells that were genotypically wild type (Fig. 4L,J). The expression of AP3 in the L3 is therefore not necessary for wild-type stamen identity.

There were a few exceptions to the above observations. Almost one third of the ap3/AP3/ap3 inflorescences showed no rescue of the mutant phenotype (Fig. 5G,H). In addition, one AP3/AP3/ap3 mosaic inflorescence lacked stamens. It is possible that the levels of AP3 expression in the L2 and L3 are critical to induce partial rescue. Those flowers that failed to form stamens may not have had sufficient levels of AP3 protein, presumably due to variable expression from the 35S promoter.

We also observed a non-autonomous effect on the maintenance of AP3 expression in the third whorl mosaic organs. In mosaics where the L1 or L2 was genetically wild-
Fig. 5. SEM micrographs of wild type and mosaic third whorl organs. (A-C) Wild-type stamens. (A) Adaxial and (B) abaxial views of a Ler stamen; the four locules are apparent. (C) Adaxial anther epidermis containing stomata. (D-F) ap3/AP3/AP3 stamens. (D) Abaxial and (E) adaxial views of the mosaic stamens. The anther and the filament are not so distinct, there are only two locules (l), and some carpeloid features are present (stigmatic papillae (arrow) and aborted ovules (arrowheads)). (F) Abaxial anther epidermis appears indistinguishable from that of a wild-type stamen. (G) Carpeloid ap3/AP3/AP3 third whorl organ (arrow). (H) Detail of the epidermis of the organ in G. The epidermis looks very similar to the epidermis of wild-type carpels (l) (v, valve; r, replum). Scale bar, 100 μm (A,B,D,E), 10 μm (C,F,H,I), 70 μm (G).

Type but not adjacent to another wild-type layer (e.g. in AP3/ap3/AP3, ap3/AP3/ap3 or AP3/ap3/ap3 flowers) we could not detect AP3 (or PI) proteins in the L1 or L2 layers of the third whorl organs (Figs 2C,D,I,J and 4E-G). Nevertheless, we did observe AP3 and PI proteins in the third whorl of flowers in which the L3 was wild type (e.g. AP3/ap3/AP3 flowers; Fig. 4E-G). This observation stands in contrast to the situation in the second whorl where we never observed any non-autonomy in the maintenance of AP3 or PI expression in the different layers.

Furthermore, we have never observed differences in protein detection in different layers or different whorls in wild-type flowers (data not shown). AP3, therefore, is required for a short-range inductive signal that is necessary to maintain AP3 and PI expression in adjacent cell layers. The non-autonomous effects of AP3 in the third whorl are clearly different from its effects in the second whorl.

These results demonstrate that AP3 acts non-autonomously in the third whorl, apparently over short distances (Fig. 7). AP3 expression in any one layer is not sufficient to confer stamen identity, but expression in both the L1 and L2 is required for normal stamen development. AP3 expression in the L2 affects the cell fate of the overlying epidermis, implying that the L2 is an important source of signals for stamen development. Signals from the L1 are also involved in organ morphogenesis, specifically in organ shape. AP3 expression is not required in the L3 for normal stamen development, suggesting that the L3 can respond to signals from the L2 and L1. Furthermore, we see a non-autonomous requirement for AP3 in the maintenance of AP3 (and PI) expression between adjacent cell layers. As in the second whorl, the AP3 protein is not transported between cells, but is localized in the cells in which it is expressed.

### Analysis of the fourth whorl ectopic stamen phenotype

In wild-type flowers, PI is expressed early (before stage 5) in the presumptive carpel primordia, but since AP3 is absent from the fourth whorl, PI expression is not maintained in that domain. When AP3 is ectopically expressed throughout the flower, AP3/PI heterodimers can form and presumably act to maintain PI expression in the fourth whorl such that stamens develop in place of carpels (Jack et al., 1994).

By analyzing mosaic sectors, we compared the specification of stamen identity in the third and fourth whorls. Specifically, we tested whether the autonomous and non-autonomous effects that we saw in the third whorl also occurred in ectopically produced stamens in the fourth whorl. In our 35SpA3G lines, the carpel to stamen transformation was partial and very variable. We classified the flowers into three classes, according to the severity of the phenotype. The first class displayed normal looking gynoecia. The second class had mild and intermediate phenotypes: the gynoecia had more than two fused carpels, which indicated a partial transformation of the carpels into stamens. The shape of the gynoecium could be distorted to various degrees (Fig. 6A). Finally, in the third, most severe, class, the fourth whorl organs appeared partially to totally unfused, and had stamen characteristics, with many organs containing locules with pollen grains (Fig. 6B).

We analyzed the phenotypes of 113 sectored inflorescences from 99 35SpA3G/+; 35S::Ac/+ plants. The results are summarized in Table 2. In these plants, a 35S::AP3/35S::AP3/35S::AP3 mosaic flower, for instance,
constitutively expresses AP3 in the L1 and L2 of the fourth whorl, but not in the L3. From examination of GUS-stained whole mounts and sectioned flowers, we can conclude that the constitutive expression of AP3 in any layer is sufficient to confer a fourth whorl phenotype consistent with a partial transformation of the carpels into stamens (Fig. 6C-E). The phenotype was more severe when AP3 was overexpressed in more than one layer, especially with overexpression in the L1 and L2 (Table 2). The aberrant shape of these fourth whorl organs in mosaic flowers could reflect the differential growth kinetics of tissues with carpel identity and tissues with stamen identity. Alternatively, the whole organ could have partial stamen identity, suggesting a non-autonomous effect of AP3 expression in any one layer. We examined $35\text{SpA3G}^{+/+}$, $35\text{SpA3C}^{+/+}$, and $35\text{SpA3G}^{+/+}$ mosaic inflorescences with anti-AP3, anti-PI and anti-GUS antibodies. We could only detect AP3 or PI proteins in the fourth whorl in the most severe cases, either in control $35\text{SpA3G}^{+/+}$ flowers (Fig. 6F,G) or in mosaic flowers (Fig. 6H,I). The observation that in severe phenotypes we see pollen locules forming in non-AP3-expressing tissues (Fig. 6H,I) supports the hypothesis that the information for stamen identity can be transferred non-autonomously from one layer to the next. In these mosaic flowers we could not find any evidence of AP3 protein movement between cell layers (Fig. 6H,I).

The ability of all layers to cause at least a partial transformation of carpels into stamens, when constitutively expressing AP3, is very different from the diverse effects of the three layers that we observed in the third whorl organs. This indicates that the fourth whorl is not equivalent to the third whorl in terms of organ identity specification, even though both whorls are expressing B- and C-class genes.

![Fig. 6.](image)

**DISCUSSION**

**Mechanisms of non-autonomy**

We have shown that AP3 acts non-cell autonomously to regulate several different processes. During petal development, AP3 expression is required in the L1 to condition normal organ shape, while its expression in the L2 is required for specifying normal second whorl organ size. Since *Arabidopsis* petals are composed of a single cell layer epidermis overlying a thin mesophyll layer (Jenik and Irish, 2000), the inter-layer cell signaling involved in coordinating petal organogenesis could be limited to short-range inductive interactions.

Further support for the role of AP3 in regulating short-range inductive interactions comes from our observations of third whorl mosaic organs. In cases where the L1 or L2 is expressing AP3 and is not adjacent to another AP3-expressing layer (e.g. in ap3/ap3/ap3, AP3/ap3/ap3, or AP3/ap3/AP3 mosaics) we have seen that those genotypically wild-type L1 and L2 layers fail to express immunologically detectable AP3 protein. Similarly, PI expression in the third whorl also appears to depend on cell-cell signaling events. This regulation could be indirect and due to the effects we see on maintenance of AP3 protein expression (Fig. 4E, G). This non-autonomy in the maintenance of AP3 and PI expression appears to be specific to the L1 and L2 in the third whorl. These observations suggest that the maintenance of AP3 expression in the third whorl relies on short-range inductive signals between cell layers.

Experiments with the maize transcription factor *KNOTTED1* (*KN1*) have shown that the KN1 protein can move between cells in the meristem (Jackson et al., 1994). Similar effects are observed with LFY in *Arabidopsis* flowers (Sessions et al., 2000). These observations led to the proposal that transcription factors could have non-autonomous developmental effects by trafficking between cells (presumably through plasmodesmata) (Mezitt and Lucas, 1996). In the mosaic flowers that we analyzed, we never observed any movement of AP3 or PI proteins into genotypically mutant layers, at any stage of organ development, in any whorl, even when non-autonomous effects were apparent. The implication from our observations is that AP3 does not traffic to other cell layers to mediate its non-autonomous effects. However, it is possible that KN1, LFY, and other transcriptional regulators exert their effects by trafficking between cells.

Since AP3 is not itself the signal, then AP3 must act by regulating a complex set of downstream genes, some of which act in a cell-autonomous fashion and some of which mediate non-autonomous effects. Only a few downstream targets of
AP3 have been identified, including *PI* and *AP3* themselves, as well as *NAP* (NAC-like, activated by *AP3/PI*), which encodes a protein of unknown biochemical function (Goto and Meyerowitz, 1994; Jack et al., 1994; Sablowski and Meyerowitz, 1998), so it is as yet unclear which signaling pathways are regulated by *AP3*. In comparison, in *Drosophila*, the homeotic genes specify cell identity in a cell-autonomous manner (McGinnis and Krumlauf, 1992; Morata et al., 1985). One exception is the non-autonomous effect of *Ultrabithorax* in the visceral mesoderm, where its signaling is mediated by *decapentaplegic* and *wingless* (reviewed by Bienz, 1994). However, comparable signaling molecules have not yet been identified in plants. Nevertheless, there is a large family of transmembrane receptors in *Arabidopsis* (Becraft, 1998), and potentially one or more of these proteins, or their ligands, could be regulated by *AP3*.

Another possibility is that biophysical signals could be involved in regulating some of these interlayer signaling events (Green, 1994). *AP3* could influence the physical properties of the cells, enabling them to grow or divide in particular orientations. The stresses created could then either mechanically mold the under- or overlying layer or activate the transcription of target genes, via mechanoreceptors. This last option is more speculative, since little is known about how physical forces at the tissue level affect organogenesis in plants.

**AP3 functions differently in the second, third and fourth whorls**

The effects of *AP3* expression are different in the three floral whorls analyzed (Fig. 7). Previous work has shown that *AP3* expression is regulated differently in the second and third whorls (Hill et al., 1998; Jack et al., 1992), and that higher levels of *AP3* expression are required for petal identity than for stamen identity (Bowman et al., 1989).

In the petals, *AP3* determines cellular identities in an autonomous way, both in the epidermis and in the mesophyll. Global aspects of petal organ identity (size and shape) are specified non-autonomously. The shape of the second whorl organs is determined by the genotype of the L1 cells, resulting in a petal-shaped organ when the epidermis is wild type or a sepal-shaped organ when the epidermis is mutant. The genotype of the mesophyll influences the size of the organ, but both a wild-type mesophyll and a wild-type epidermis are needed for a full sized petal. Similarly, in chimeric tobacco flowers, the L1 autonomously controls aspects of epidermal cell identity, while the L1, L2 and L3 all contribute to determining petal size (Kaddoura and Mantell, 1991; Marcotrigiano, 1986).

In the third whorl there is also evidence for non-autonomous effects of *AP3*: a mutant L2 conditions a mutant organ, but a mutant L3 has no effect on organ identity. As in the petals, the L1 seems to act non-autonomously in providing much of the information controlling the shape of the organ. However, contrary to the situation in the petals, the specification of cellular fate in the epidermis is non-autonomous and depends on the genotype of the L2. All these observations point to the L2 as the main source of *AP3*-regulated signals in the specification of stamen identity. By the same token, though, *ap3/AP3/ap3* mosaic flowers show no rescue of the third whorl phenotype. This could be due to the requirement for interlayer signaling to maintain *AP3* expression in the L2 of the third whorl, and in these *ap3/AP3/ap3* flowers such signaling is not present. The distinctions observed in the mosaics of the second and third whorls suggest that *AP3* regulates cell-cell interactions through different mechanisms during petal and stamen development. These differences in *AP3* function could be context dependent; it is possible that *AP3/PI* heterodimer binding to target sequences could be modulated by interactions with whorl-specific ternary factors. The products of the orthologous *Antirrhinum DEF* and *GLO* genes form a ternary complex with the product of the *SQUA* MADS box gene which affects DNA binding properties, providing biochemical support for such a model (Egea-Cortinés et al., 1999).

Surprisingly, specification of stamen identity in the fourth whorl, caused by ectopically expressing *AP3*, seems to operate differently than in the third whorl. In the fourth whorl, any layer overexpressing *AP3* is capable of providing at least some degree of stamen identity to the organ, and the effect of expressing *AP3* in the different layers is mostly additive. Even though both the third and fourth whorls are expressing B- and C-class homeotic genes in these mosaics, there are apparent differences in the response of the whorls to *AP3* expression. It is possible that as yet uncharacterized genes are expressed differentially in these two whorls, in a manner independent of organ identity. The presence or absence of such gene expression would give the layers in the different whorls distinct competence to generate, or respond to, *AP3*-regulated signals.

**Do all floral organ identity genes utilize the same signaling mechanisms?**

The *Arabidopsis LFY* gene and the orthologous *Antirrhinum*
The Arabidopsis AGAMOUS (AG) gene also acts in a non-cell autonomous manner to regulate third and fourth whorl development (Sieburth et al., 1998). In the third whorl the effects of AG are similar to what we have observed for AP3; in that expression in the L2 is required for stamen identity and for epidermal cell type differentiation, while expression in the L3 is not required for organ identity. One possibility for these similar effects by AP3 and AG in the third whorl is that both genes are co-regulating the same set of target genes for the specification of organ identity. In fact, both AP3/PI and AG interact in vitro with SEPALLATA3, which is required for petal, stamen and carpel identity and which may play a role in regulating the genes that mediate these non-autonomous effects (Fan et al., 1997; Pelaz et al., 2000).

In the case of DEF, the Antirrhinum orthologue of AP3, mosaic analyses indicate that DEF and AP3 function in a similar manner to regulate organogenesis (Perbal et al., 1996). In the second whorl, both AP3 and DEF specify cell type identity in a cell-autonomous fashion, and both of them have non-autonomous effects on petal growth and shape. In the third whorl, the expression of DEF in the L1 is insufficient to produce stamens, and its expression in the L2+L3 produces staminoid organs that do not have the appropriate shape, similar to what we observe for the AP3 mosaic. In contrast to the situation with AP3, though, the DEF protein has been reported in the L1 of a def/DEF/DEF mosaic (Perbal et al., 1996). However, the movement of the DEF protein into the L1 did not have any apparent phenotypic consequences in that situation with AP3, though, the DEF protein has been reported in the L1 of a AP3/ap3/ap3 mosaic (Perbal et al., 1996). We did not observe trafficking of the DEF protein into the L1 of a AP3/ap3/ap3 mosaic (Perbal et al., 1996). However, the movement of the DEF protein into the L1 did not have any apparent phenotypic consequences in that case. Mosaic flowers did not display a wild-type phenotype.  

In contrast to the reported observation that the product of the Antirrhinum orthologue of PI, GLO, traffics between cell layers (Perbal et al., 1996), we did not observe trafficking of the PI protein. In our AP3 mosaics, the distribution of PI protein was always coincident with that of AP3 protein after stage 5. AP3/ap3/ap3 mosaic plants show a restriction of PI protein to the L1, yet still display a partially rescued second whorl and a mutant third whorl (Table 1; Fig. 2B-F). The restriction of PI protein to genetically wild-type cell layers in our mosaics also suggests that PI mediates its effects, presumably as part of an AP3/PI heterodimer, by regulating the production of intercellular signaling molecules. Evidence suggesting that PI expression in the L1 can influence the underlying layers has been previously reported (Bouhidel and Irish, 1996).


