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Regulation of cell proliferation patterns by homeotic genes during *Arabidopsis* floral development

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SUMMARY

The shoot apical meristem of *Arabidopsis thaliana* consists of three cell layers that proliferate to give rise to the aerial organs of the plant. By labeling cells in each layer using an *Ac*-based transposable element system, we mapped their contributions to the floral organs, as well as determined the degree of plasticity in this developmental process. We found that each cell layer proliferates to give rise to predictable derivatives: the L1 contributes to the epidermis, the stigma, part of the transmitting tract and the integument of the ovules, while the L2 and L3 contribute, to different degrees, to the mesophyll and other internal tissues. In order to test the roles of the floral homeotic genes in regulating these patterns of cell proliferation, we carried out similar clonal analyses in *apetala3-3* and *agamous-1* mutant plants. Our results suggest that cell division patterns are regulated differently at different stages of floral development. In early floral stages, the pattern of cell divisions is dependent on position in the floral meristem, and not on future organ identity. Later, during organogenesis, the layer contributions to the organs are controlled by the homeotic genes. We also show that *AGAMOUS* is required to maintain the layered structure of the meristem prior to organ initiation, as well as having a non-autonomous role in the regulation of the layer contributions to the petals.

Key words: *Arabidopsis thaliana*, Floral meristem, Cell division, Homeotic gene, *AGAMOUS*, *APETALA3*, Clonal analysis

INTRODUCTION

Plant cells are surrounded by a rigid cell wall that binds them together and that prevents them from moving or migrating. Plants therefore utilize a fine control of the rates and planes of cell divisions during development to generate organs in the appropriate numbers and positions, with a characteristic size and shape (Meyerowitz, 1997; Steeves and Sussex, 1989). One way to analyze how these processes are regulated is by studying the patterns of cell division during development and how they are affected in mutants that alter the number, size or shape of the organs. All the aerial organs of flowering plants are generated by a group of undifferentiated cells, the shoot apical meristem (SAM). The analysis of chimeras has shown that the SAM is composed of three lineages of cells termed 'histogenic layers' (L1, L2 and L3) (Satina et al., 1940). These 'layers' have also been described at the morphological level: the tunica (L1 and L2) comprises the outermost layers of cells of the SAM which generally only divide anticlinally, while the internal corpus (L3) cells divide in all directions, increasing the volume of the meristem (Tilney-Bassett, 1986) (Fig. 1A). The control of the orientation of cell divisions prevents the mixing of the layers during ontogenesis, although on rare occasions cells inappropriately divide into other layers (Stewart and Dermen, 1970). After the induction of flowering both the inflorescence and the floral meristems (IM and FM, respectively) maintain the same layered structure, with each layer deriving from the corresponding one in the SAM (Tilney-Bassett, 1986; Vaughan, 1955).

In order to understand how normal floral development proceeds and how mutations affect the development of particular structures, detailed analyses of morphology as well as the dynamics of cell proliferation are necessary. Clonal analyses and fate mapping studies have been carried out for the *Arabidopsis* root, and have been of fundamental importance in interpreting how mutations or physical perturbations affect the root (Dolan et al., 1994; Scheres et al., 1994). However, despite the wealth of genetic and molecular data on the regulation of flower development in *Arabidopsis*, there has been little comparable fate mapping information for the flower. The contributions of marked clones to particular *Arabidopsis* floral tissues have been assessed in a few studies (Bossinger and Smyth, 1996; Bouhidel and Irish, 1996; Furner and Pumfrey, 1993), but no comprehensive survey of the contributions of each histogenic layer to all of the floral structures has been reported. In other dicots, clonal analyses in the flower indicate that the contributions of the histogenic layers to the floral organs differ significantly between species (Blaser and Einset, 1950; Dermen, 1947, 1953; Dermen and Stewart, 1973; Satina, 1944, 1945; Satina and Blakeslee, 1941, 1943; Stewart et al., 1974). Establishing how many histogenic layers are present in *Arabidopsis*, what are their contributions to the floral organs

and how these contributions are regulated is necessary to lay the foundation for a detailed understanding of the effects of mutations on floral development in this species. To this end we constructed a detailed fate map of the wild-type flower and evaluated the plasticity of floral development. We found that whereas, in general, the contributions of the different cell lineages to the mature flower are similar in *Arabidopsis* to those of other species, the *Arabidopsis* floral fate map also shows a number of unique features.

The layer contributions to the floral organs in all the species studied are fairly stereotypical, but it has also been well established that the final fate of a cell depends on its final position and not on its lineage. When a cell divides in the 'wrong' direction, and its daughter cell moves to another layer, the development of the plant still proceeds normally (Stewart and Dermen, 1970; Stewart et al., 1974). These observations generate a number of questions. What are the genetic programs that determine the specific contributions of the different lineages to the floral organs? Are these cell divisions regulated by the position of an organ in the flower or by its identity? The *Arabidopsis* floral homeotic mutants are good candidates to address these questions. Plants mutant for *APETALA3* (*AP3*) have petals that are transformed into sepals, and stamens into carpels (Jack et al., 1992). Plants mutant for *AGAMOUS* (*AG*) are affected in organ identity such that stamens are transformed into petals. Mutations in *AG* also disrupt the regulation of cell proliferation, in that instead of a pistil, an indeterminate number of nested flowers are formed (Bowman et al., 1989). We used these mutant backgrounds to examine the relationships between organ identity and position and the control of cell division patterns. Using a cell marking strategy, we conducted a clonal analysis in each of these mutant backgrounds. In both *ap3-3* and *ag-1* mutants we found that cell divisions are regulated differently at early and at late stages during floral development. Until floral stage 6 the pattern of cell divisions is dependent on the radial position of the organ primordia in the meristem, and is identical to the wild type. Later on, the homeotic genes control the rate and orientation of cell divisions, resulting in meristematic layer contributions that reflect the identity of the organ. In addition, we uncovered two previously undescribed roles for *AG*: in preventing inappropriate divisions of the L2 in the developing floral primordia and in controlling the relative amounts of L2 and L3 contributions to the petals. These observations suggest a non-autonomous role for *AG* in regulating the patterns of cell division in the developing flower.

MATERIALS AND METHODS

Plant material

The pBI35SAc11-5.1 line (Fig. 1B) was a gift from David Smyth (Monash University, Australia) (Bossinger and Smyth, 1996). To generate sectors in mutant backgrounds, the pBI35SAc11-5.1 line was crossed to *apetala3-3* homozygotes or *agamous-1* heterozygotes and the F_2 generation was analyzed. Plants were selected on kanamycin and then transplanted onto a 12:3:1 vermiculite:soil:sand mixture and grown under long day conditions.

Histochemistry and immunohistochemistry

To detect GUS activity, inflorescences were stained overnight at 37°C in 1 mg/ml X-glucuronic acid, 1 mM EDTA, 1% Tween-20 and 1-5 mM potassium ferri/ferrocyanide in 100 mM phosphate buffer pH 7. The material was destained in 70% ethanol and either studied under a dissecting microscope or dehydrated, embedded in paraffin wax (Tissue-Prep 2; Fisher Scientific, Fair Lawn, NJ), cut in 8 µm sections and analyzed using dark-field optics on a Zeiss Axiophot microscope.

To detect GUS protein on tissue sections, four flowers per inflorescence were histochemically stained. Inflorescences that showed sectoring were cut and fixed overnight at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS). The material was then dehydrated, embedded in paraffin wax and sectioned. Sections were rehydrated and treated for 10 minutes at room temperature with 10 µg/ml proteinase K in PBS, then washed three times in PBT (PBS + 0.1% Tween-20). After blocking for 1 hour at room temperature in 0.5% BSA, 1% goat serum in PBT, the slides were incubated with a 1/1000 dilution of anti-GUS antibody (Molecular Probes, Eugene, OR) in PBT + 0.5% BSA for 5 hours at 4°C. The slides were then washed three times in PBT and incubated overnight at 4°C with a 1/2000 dilution of the secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit; Jackson ImmunoResearch, West Grove, PA). The slides were subsequently washed three times in PBT and two times in TNM (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) and developed with 0.33 mg/ml 4-nitroblue tetrazolium and 0.16 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim, Indianapolis, IN) in TNM. Sections were examined under bright-field or DIC optics. Images were taken on Kodak slide film and assembled using Adobe Photoshop (Adobe Systems, Mountain View, CA).

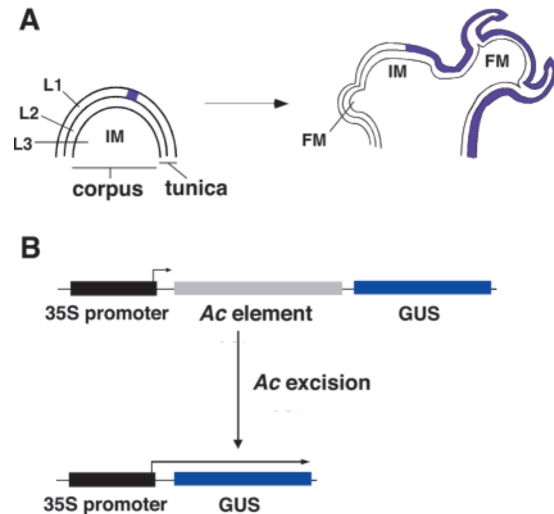


Fig. 1. The experimental system. (A) Structure of the inflorescence meristem (IM) and generation of a sectored plant by labeling one cell. After proliferation, one floral meristem (FM) is not sectored (left), while the other is a periclinal mosaic. (B) The 35S::Ac::GUS transgene used in this study. Excision of the *Ac* element leads to reconstitution of GUS expression.

RESULTS

The experimental system

In order to obtain sectored plants, we used a transgenic line carrying the Cauliflower Mosaic Virus 35S promoter and the β-glucuronidase (GUS) marker gene, separated by a modified *Ac* transposable element (pBI35SAc11-5.1; Fig. 1B; Bossinger and Smyth, 1996). In these plants the transposon will excise at random, generating a clone of cells that expresses GUS under the control of the 35S promoter in a cell-autonomous fashion.

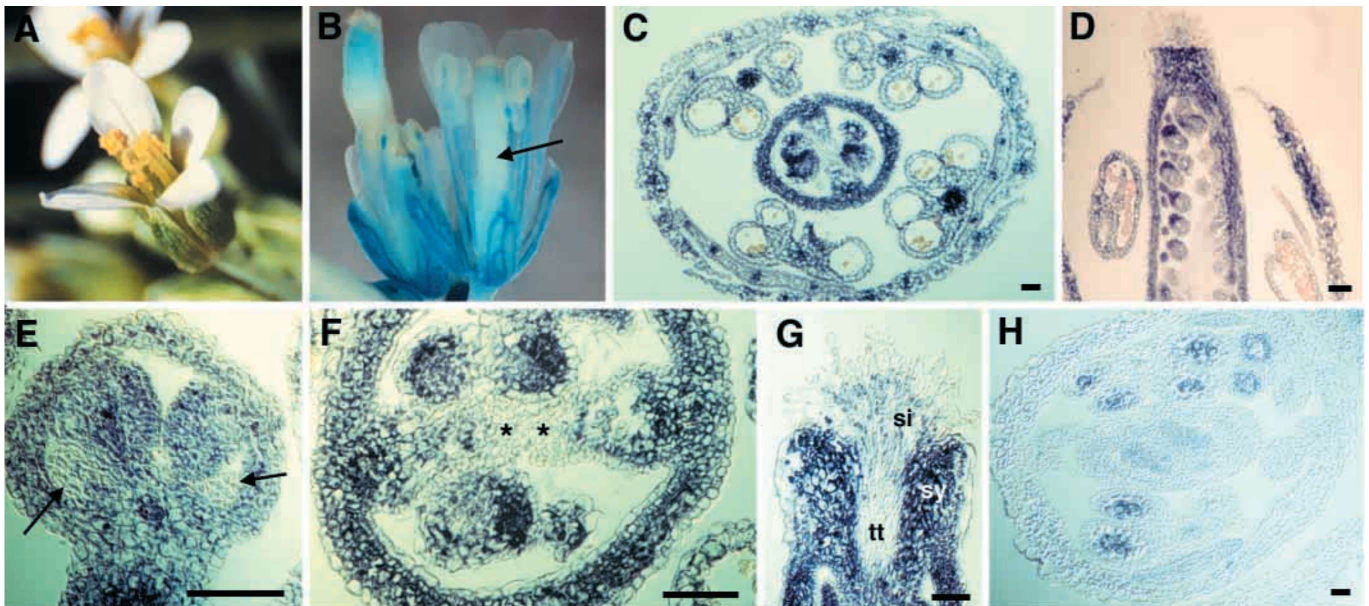


Fig. 2. Pattern of expression of the 35S promoter in the flower. (A) Mature wild-type flower. (B-G) *35S::GUS* flowers. (B) Flower stained with X-Gluc. Note weak staining in the carpel (arrow). (C-H) Flower sections probed with anti-GUS antibody. Sections C,F and H are transverse; sections D,E and G are longitudinal. (C,D) Mature flower. Most cells express GUS protein, including in the carpel. (E) Stage 8 flower, the petal primordia show low levels of expression (arrows). (F) Higher magnification of flower shown in C. The cells of the transmitting tissue of the ovary do not express GUS (asterisks). (G) Style (sy) and stigma (si) of a stage 13 flower. The transmitting tissue of the style (tt) shows no GUS expression at this stage, but the stigma does. (H) Non-transgenic flower probed with anti-GUS antibody as a negative control. Scale bars, 100 μm.

If the excision occurs in a cell of the SAM or IM, then a part or a whole cell layer and its derivatives will be labeled (mericlinal or periclinal mosaics, respectively; Fig. 1A). Since cells expressing GUS do not have any apparent growth disadvantage, the distribution of clones should reflect the developmental dynamics of the normal flower (Stewart et al., 1974). We obtained a high frequency of early sectors, usually one per plant. The frequency of germinal excisions, as measured by the percentage of completely blue seedlings produced by 40 non-sectored flowers, was 25% (283/1116 seedlings), ranging from 5% to 40% per flower. Similar frequencies have been reported for *Ac* transposons in which the 35S promoter drives the transposase (Grevelding et al., 1992).

Pattern of expression of the 35S promoter in the flower

The transgenic plant line used in this study contains a commonly used version of the 35S promoter driving GUS (Jefferson et al., 1987). This promoter has been widely viewed as constitutive, but previous studies involving analyses of phenotypes (Jack et al., 1994) or staining for GUS activity (Bossinger and Smyth, 1996; Sieburth et al., 1998; Wilkinson et al., 1997) suggest that expression driven by this promoter is not uniform in the flower and that it may show variations during development. For our study we first needed to characterize this expression in detail. We stained whole inflorescences of 35S::GUS plants with X-Gluc to detect GUS activity (Fig. 2A,B). These plant lines were derived as germinal excisions of the *Ac* element from pBI35SAc11-5.1 (Fig. 1B) (Bossinger and Smyth, 1996). Most of the cells of the transgenic flowers stained for GUS but, using several staining conditions, the intensity of the signal was unpredictable. Probing tissue sections with an anti-GUS antibody gave more consistent results.

Almost every cell of the flower expressed GUS, including most cells of the mature carpel (Fig. 2C,D). Early expression

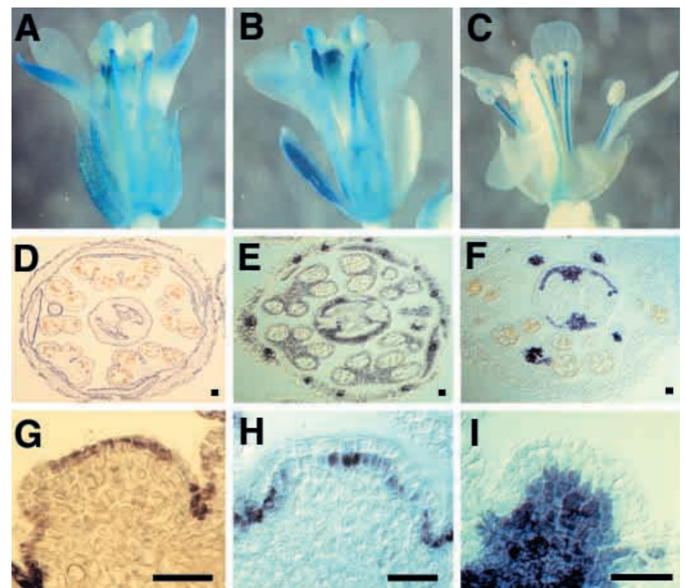


Fig. 3. Three independent cell layers in *Arabidopsis*. (A-C) Mature flowers stained with X-Gluc, and (D-F) transverse sections probed with anti-GUS antibody, showing the three different patterns of staining: labeled L1 (A,D), L2 (B,E) or L3 (C,F). (G-I) Longitudinal sections through inflorescence meristems, probed with anti-GUS antibody, showing a single labeled layer: L1 (G), L2 (H), L3 (I). Scale bars, 50 μm.

(until stage 7; stages as defined by Smyth et al., 1990) was variable in intensity, and the levels of GUS expression in some organs varied with the developmental stage of the flower (this was also observed in histochemically stained tissue). Petals showed very low levels of expression until stage 8 (Fig. 2E). This is consistent with the fact that mutations that affect the petals are not completely rescued when the expression of the wild-type allele is driven by the 35S promoter (Jack et al., 1994). Other tissues that showed lower levels of expression include the stamen primordia (only at stages 5 and 6), the epidermis in general, the ovule primordia at their inception (stage 9), and the embryo sac (data not shown). The transmitting tissue of the ovary did not show any GUS expression (Fig. 2F), but the cells that give rise to the transmitting tissue in the style continued to express GUS until anthesis (stage 13). After that, GUS protein was no longer detectable in this tissue (but it was still present in the stigma) (Fig. 2G). We did not observe any anti-GUS antibody signal in the tapetum cells or in mature pollen grains in the anther, despite high levels of X-Gluc histochemical staining, suggesting that the GUS epitopes are masked in these tissues. The same results were observed in several independent 35S::GUS transgenic plant lines. We did not detect any GUS protein or activity in control inflorescences (data not shown).

Table 1. Number of flowers (inflorescences) analyzed for this study

Genotype Layer(s) expressing GUS	Analyzed by staining for GUS activity and sectioning	Analyzed by probing with anti-GUS antibody	Total
35S::GUS	68 (5)	80 (6)	148 (11)
Wild type; 35SAcGUS			
L1	12 (2)	37 (3)	49 (5)
L2	23 (3)	33 (4)	56 (7)
L3	35 (6)	47 (4)	82 (10)
L1+L2	12 (2)	0	12 (2)
L2+L3	0	34 (3)	34 (3)
L1+L3	9 (1)	0	9 (1)
ap3-3; 35SAcGUS			
L1	0	32 (3)	32 (3)
L2	0	21 (2)	21 (2)
L3	0	45 (3)	45 (3)
L1+L2	0	8 (1)	8 (1)
L2+L3	0	33 (2)	33 (2)
L1+L3	0	31 (2)	31 (2)
ag-1; 35SAcGUS			
L1	0	48 (3)	48 (3)
L2	0	37 (2)	37 (2)
L3	0	51 (3)	51 (3)
L2+L3	0	21 (2)	21 (2)

Clonal analysis of the wild-type flower

The SAM, IM and FM of *Arabidopsis* contain a two layered tunica and a corpus, as in other dicot plants (Vaughan, 1955). To study the relative contributions of each of the meristematic layers to the flower, we screened inflorescences by staining for GUS activity, and also by probing with the anti-GUS antibody. We only analyzed inflorescences that were mericlinal or periclinal mosaics. We detected only three basic staining patterns in whole flowers (Fig. 3A-F). These patterns correspond to the labeling of cells derived from each of the three histogenic layers (Fig. 3G-I), demonstrating that these are clonally distinct layers in *Arabidopsis.* The number and genotypes of flowers used in this study are summarized in Table 1.

The perianth

The perianth comprises the sterile organs of the flower: four sepals (first whorl) and four petals (second whorl). Both organ types have a simple laminar structure, consisting of an epidermis and a mesophyll. The petal mesophyll extends all the way to the edge of the organ, while in the sepals the edges consist of juxtaposed abaxial and adaxial epidermal cells. Both sepal and petal primordia are initiated by periclinal divisions in the subepidermal cell layer (Hill and Lord, 1989). The sepal primordia arise at stage 3 and the petal primordia at stage 5. Both sets of primordia grow by periclinal and oblique cell divisions in the presumptive mesophyll and by anticlinal cell divisions in the epidermis (Hill and Lord, 1989). In agreement with the morphological description, we find that the L1 contributes to the epidermis only, even at the organ margins (Fig. 4A). The mesophyll of both organ types derives from the L2 (Fig. 4B). We have also observed that 23% (9/46) of the time, the L3 contributes to the vasculature in the basal part of the sepals (Fig. 4C), but we have never detected any L3 contribution to the petals.

The stamens

The third whorl contains six stamens, each composed of a filament and an anther. The stamen primordia are initiated at stage 5 by periclinal divisions in the subepidermal cell layer (and sometimes in the L3; Crone and Lord, 1994). By stage 7 they become stalked, and at stage 8 the locules become apparent (Smyth et al., 1990). The sporogenous tissue is first visible at stage 9 in the center of the locules (Sanders et al., 1999). Our analysis reveals that the cells in the L1 divide only anticlinally, giving rise to the epidermis of both the filament and the anther (Fig. 4D). At stage 7 the stamen primordia are composed of an L1-derived epidermis, one layer of L2-derived subepidermis and an L3-derived core. The growth of the internal anther tissues after stage 8 is due mostly to the division of L2-derived cells in different planes. These cells will give rise to the connectives, endothecium, sporogenous tissue and tapetum (Fig. 4E). In the mature anther, the L3 contributes only to the vasculature (Fig. 4F). These results are consistent with the pattern of cell divisions observed by Sanders et al. (1999) in the developing anther. We have observed some variability in the contributions to the connectives. In 46% of the stamens (37/80) the L3 divides at the expense of the L2 and contributes to the connectives, usually on only one side of the anther (Fig. 4G). The mature filament is composed of an L1-derived epidermis, an L2-derived subepidermal layer and an L3-derived core.

The gynoecium

The fourth whorl of the flower, the gynoecium, or pistil, is formed by two congenitally fused carpels. The mature gynoecium can be divided into a basal ovary, which contains the ovules in two locules separated by a false septum, and a distal style, which is topped by the stigma. The gynoecium arises at stage 6 as a circular rim of tissue in the center of the floral meristem, due to periclinal cell divisions in the L3. The primordium grows initially as an open cylinder by anticlinal cell divisions in the epidermis and subepidermis (Hill and Lord, 1989). By early stage 8, the cylinder is composed of an L1-

derived epidermis, one L2-derived subepidermal layer and a 2-cell thick, L3-derived, core. Beginning at stage 8 the distal L2 cells start to divide periclinally, contributing to the longitudinal growth of the carpel (Fig. 4H). The mature ovary wall is six cells thick. The outer and the inner epidermis (the latter lines the cavity of the locules) are derived from the L1 (Fig. 4I). The intervening layers derive from the L2 and L3. The relative contribution of the L2 and the L3 to the carpel wall is very variable and differs from flower to flower, even from carpel to carpel in the same pistil. In general, the base of the ovary is composed of a single L2-derived subepidermal layer, with the rest of the ovary wall deriving from the L3 (Fig. 4J). Towards the style, the L2 contribution to the internal layers increases, and the L3 contribution decreases progressively to just the base of the septum on each side (Fig. 4K). The L3 contribution to the gynoecium usually terminates one half to three quarters of the way up the ovary wall (Fig. 4L) (Bouhidel and Irish, 1996). This variability in the amount of mesophyll derived from the L2 and L3 has been observed in the carpels of other species (Dermen, 1947; Dermen and Stewart, 1973; Satina, 1944). The false septum runs the length of the ovary. It starts growing at stage 8, by periclinal divisions in the L2 and L3. These outgrowths will eventually fuse postgenitally (Sessions, 1997). The base of the septum is made of L3-derived cells, while the rest of the structure (including the cells that will differentiate as the ovary transmitting tissue; Sessions and Zambryski, 1995) is derived from the L2. The only exception is a two-cell thick L1-derived 'scar' at the plane of fusion (Fig. 4M). The style is a solid cylinder composed of L2-derived cells, covered by an L1-derived epidermis (Fig. 4N). The center of the style is composed of files of elongated cells that form the beginning of the transmitting tissue and that are a continuation of the stigma (Sessions and Zambryski, 1995). Both the stigma and the transmitting tissue of the style are L1-derived (Fig. 4O). The transmitting tissue of the style arises by periclinal divisions of the inner epidermis of the carpel prior to the postgenital fusion (Sessions, 1997). It is interesting to note that the stigma and the transmitting tissue of the style derive from the L1, but that the transmitting tissue in the ovary is derived from the L2.

The pistil contains the ovules (for a detailed description of ovule development see Schneitz et al., 1995). The ovules are borne on the placenta, where the septum contacts the ovary wall. The placenta is composed of cells derived from the L1 and L2 or from all three layers, depending on the position in the pistil. The ovules arise as finger-like primordia formed by divisions in the subepidermal layer at stage 8. At stage 10 a cell in the epidermis (derived from the L1) on each side of the primordium divides periclinally or obliquely to give rise to the inner integuments (Fig. 4P). The outer integuments arise a little later in a similar fashion. The distalmost subepidermal cell (derived from the L2) differentiates as the megaspore mother cell, and will eventually give rise to the female gametophyte (Fig. 4Q). The mature ovule has tissues derived from both L1 and L2. The L1 gives rise to the epidermis of the funiculus (ovule stalk) and to the integuments (Fig. 4R). The L2 contributes to the center of the funiculus, to the chalazal region and to the embryo sac (Fig. 4S). We have also observed that the L3 gives rise to the vasculature of the funiculus in some ovules (Fig. 4T).

Other structures

The receptacle (base of the flower), the pedicel and the inflorescence stem are composed of an L1-derived epidermis, a single-cell thick subepidermal L2 layer and a thick L3-derived core (data not shown).

Table 2. Number of organs examined for the layer invasion study

	Sepals	Petals	Stamens	Pistils
L1 sector				
No invasion	48	48	65	13
L2 to L1 invasion	0	0	0	0
L2 sector				
No invasion	57	64	43	15
L1 to L2 invasion	1	0	0	6
L3 to L2 invasion	*	*	37	‡
L3 sector				
No invasion	*	*	100	‡
L2 to L3 invasion	*	*	5	‡
L2+L3 sector				
No invasion	80	80	129	13
L1 to L2 invasion	0	0	0	7
Total	232	192	379	54

Periclinal sectors expressing GUS in different cell layers were assessed for unstained cells invading that layer.

*The L3 does not normally contribute to the sepals and the petals.

‡The L2 and L3 contributions to the pistils are very variable.

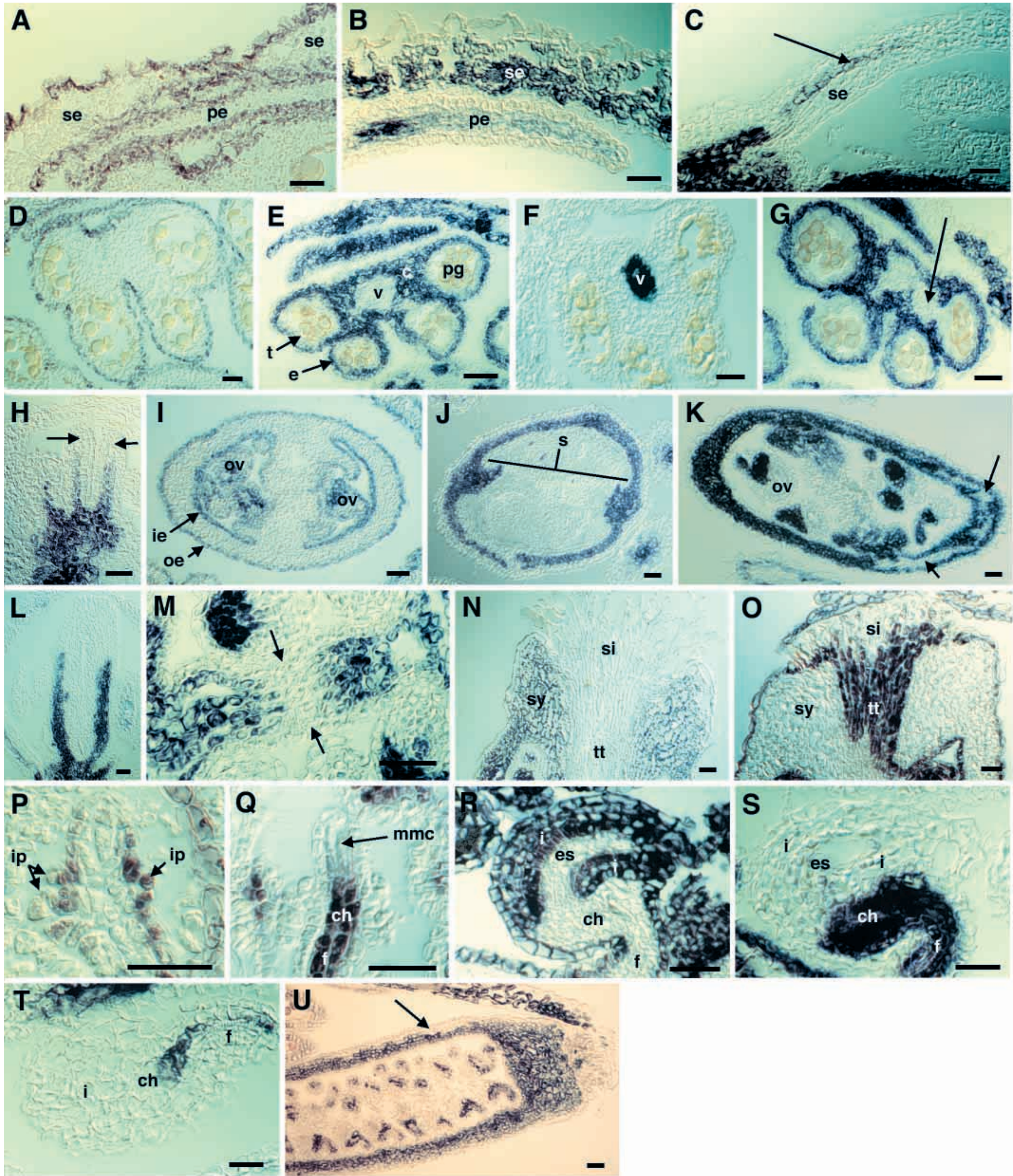
Layer invasions and plasticity in floral development

The cell lineages derived from the three meristematic layers remain independent during the life of the plant. Since the final fate of the cells is dependent on their position, not their lineage, the occasional layer invasion by the division of a cell in a non-habitual direction will not affect the development of the plant (Stewart and Dermen, 1970). To analyze how common these non-habitual cell divisions are during *Arabidopsis* development, we examined individual organs in tissue sections probed with anti-GUS, looking for invasion of unlabeled cells into labeled sectors. The converse experiment (looking for invasion of labeled cells into unlabeled sectors) would have been difficult to interpret, because it would not have been possible to distinguish a layer invasion from a secondary excision event.

We analyzed a total of 232 sepals, 192 petals, 379 stamens and 54 pistils, in flowers of stages 10 and later (Table 2). There is a range of frequencies of layer invasions. In some tissues it is very high, for instance in the L2 and L3 in the ovary walls and the connectives of the anthers, or L3 invasions into the base of the sepals (see above). In other tissues layer invasions are very rare. We did not observe any invasions before the primordia of the floral organs appeared, nor did we see inappropriate divisions into the L1 in any organ. Aberrant divisions of the L2 into the L3 or the L1 into the L2 occurred very sporadically, and were usually limited to a small area (Fig. 4U). These results suggest that in a few tissues the pattern of cell divisions is more plastic, and that in others it is much more constrained. In *Arabidopsis*, therefore, the contributions of the meristem layers to the floral organs are very stereotypical, with limited variability only in some of the internal tissues of the sepal, stamen and carpel.

Clonal analysis of the *ap3-3* mutant

The *AP3* gene is required to specify petal and stamen identity and encodes a MADS-domain DNA-binding protein (Jack et



al., 1992). *AP3* is expressed starting at stage 3 in the areas of the FM that will give rise to the second and third whorl organs, and later throughout the petals and stamens until maturity. Null alleles (such as *ap3-3*) produce a homeotic transformation of the petals into sepals and the stamens into carpels (Fig. 5A). The second whorl organs show a consistent transformation to sepals. The appearance of the third whorl organs is much more variable: they range from being absent, to filaments, to filaments capped with stigmatic tissue, to carpeloid organs, which are often fused to the central gynoecium.

Fig. 4. Clonal analysis of the wild-type flower. Sections of flowers with one meristematic layer labeled, probed with anti-GUS antibody. Sections A,B,D-G,I-K and M are transverse; sections C,H,L and N-U are longitudinal. (A,B) Sepals (se) and petals (pe). The L1 contributes to the epidermis (A) and the L2 to the mesophyll (B). (C) A sepal showing L3 contribution (arrow). (D-G) The anthers. (D) The L1 contributes to the epidermis, (E) the L2 to the connectives (c), endothecium (e), tapetum (t) and pollen grains (pg), and (F) the L3 to the vasculature (v). (G) In a number of stamens, the L3 also contributes in part to the connectives (arrow). (H) Stage 8 flower with a labeled L3. The distal-most L2 cells of the carpel have started dividing periclinally (arrows). (I-K) Late stage ovaries. (I) The L1 contributes to the epidermis (inner, ie, and outer, oe) and part of the ovules (ov). (J) In the basal part of the pistil, the L3 (labeled) makes up most of the ovary wall and the base of the septum (s). (K) More distally, the L2 (labeled) contributes to most of the carpel wall and also to the ovules (ov) with little L3 contribution (arrows). (L) Stage 11 flower showing the vertical extent of the contribution of the labeled L3 to the carpel wall. (M) The septum of an L2-labeled stage 11 flower. The L1 'scar' is visible at the plane of fusion (arrows). (N,O) The style and stigma. (N) The L2 contributes to the mesophyll of the style (sy) and (O) the L1 to the epidermis, the stigma (si) and the transmitting tissue (tt). (P,Q) Ovule primordia. (P) The L1 gives rise to the epidermis and integument primordia (ip). (Q) The L2 contributes to the funiculus (f), chalaza (ch) and megaspore mother cell (mmc). (R-T) Mature ovules showing the contributions of the L1 (R), the L2 (S) and, occasionally, the L3 (T). i, integuments; es, embryo sac. (U) Stage 12 carpel with a labeled L2. An L1 into L2 invasion is apparent (arrow). Scale bars, 50 μm.

We performed a clonal analysis in *ap3-3* mutants, to assess whether the contributions of the meristematic cell layers to the floral organs were determined by the position of the organ or by its identity. There were no differences in *ap3-3* mutants with respect to the wild type in the structure of the IM. We could not detect any differences between the wild type and the mutant in the contributions of the histogenic layers to the floral organ primordia until stage 6 (data not shown). In the mature *ap3-3* flower, the second whorl organs presented a typical sepal structure: an L1-derived epidermis, an L2-derived mesophyll and, in a feature that distinguishes sepals from petals, the participation of the L3 in the formation of the vasculature in the basal portion of some of these organs (Fig. 5B). Starting at stage 7 it became obvious that no stamens were present but instead there were extra filaments or carpeloid organs, sometimes fused to the fourth whorl. These structures were composed, in many cases, of cells derived from all three layers (Fig. 5C). At maturity, the third whorl filaments were covered in an L1-derived epidermis and their interior was variably composed of cells derived from the L2 only or the L2 and L3 (Fig. 5D). When the transformation to a carpel was more complete, the third whorl organs showed a distribution of cell layers identical to that of wild-type carpels in all aspects. This was the case whether the organ was free standing (Fig. 5E) or fused to the central gynoecium (Fig. 5F,G,H). There was no L1-derived 'scar' tissue in fused organs (Fig. 5F,I), supporting previous observations that these fusions are congenital (Jack et al., 1992). The ovules in the extra carpels also showed a wild-type layer contribution (data not shown). We conclude that second whorl 'sepals' and third whorl 'carpels' show a layer structure corresponding to that of sepals and carpels and not to that of wild-type second and third whorl organs. In summary, the layer contributions to the primordia in *ap3-3* mutants are indistinguishable from the contributions to the wild-type primordia that normally appear at that position up until stage 6. After that point, the organ develops with the cell division and growth patterns typical of its transformed identity.

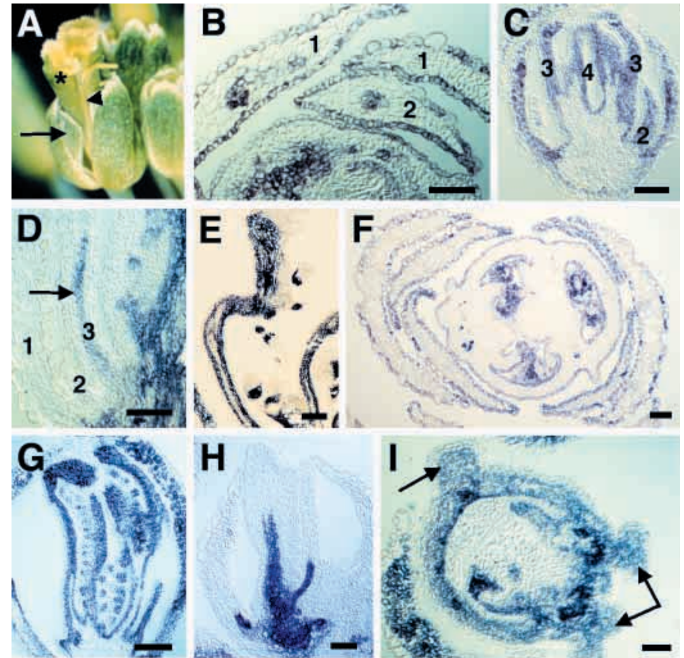


Fig. 5. Clonal analysis of the *ap3-3* mutant. (A) *ap3-3* flower. Note the second whorl sepals (arrow) and the third whorl filaments (arrowhead) and fused carpel (asterisk). (B-I) Sections of *ap3-3* flowers probed with anti-GUS antibody. Sections B, F and I are transverse, sections D, E, G and H are longitudinal, and section C is oblique. (B) Flower in which the L1 and the L3 are labeled. The L3 contributes to both the first (1) and the second (2) whorls. (C) Stage 9 flower, with the L2 labeled, showing the growth of abnormal organs in the third whorl (3), fused to the fourth whorl (4). The second whorl is already morphologically sepaloid (2). (D) L3-labeled flower, showing the contribution of this layer to a third whorl (3) filament (arrow). (E-H) Flowers labeled in the L1 (F), L2 (E,G) or L3 (H). Here the third whorl organs are completely transformed into carpels, and they show a wild-type carpel layer contribution, whether they are free-standing (E) or fused to the fourth whorl (F-H). (I) Flower labeled in the L2 and L3, in which third whorl filaments have fused to the carpels (arrows). No L1 'scar' is apparent (see also F). Scale bars, 100 μm.

Clonal analysis of the *ag-1* mutant

The *AG* gene is necessary for the specification of carpel and stamen identity and is required for determinate development of the flower (Bowman et al., 1989). *AG* encodes a MADS-domain protein that is initially expressed at stage 3 in the presumptive third and fourth whorls, and maintained in this region as stamens and carpels develop (Drews et al., 1991). The flowers of *ag-1* (a severe allele) display a homeotic transformation of the stamens into petals. In addition, the carpels are replaced by an indeterminate number of nested flowers that have a whorl sequence of sepal-petal-petal (Fig. 6A) (Bowman et al., 1989). We performed a clonal analysis in this mutant background to determine the layer contributions to these extra organs, which have no counterpart in the wild-type flower. In *ag-1* the third

and fourth whorl organ primordia are initiated as in the wild-type flower, but they develop as petals or sepals (Crone and Lord, 1994). The transformed third whorl and the organs of the internal flowers of *ag-1* showed a contribution of layers corresponding to their transformed identity. Like sepals or petals, they were composed of an L2-derived mesophyll covered by an L1-derived epidermis (Fig. 6B-E). These results further support the hypothesis that the patterns of cell divisions in a developing organ depend on its final identity.

We also observed two unexpected phenotypes in *ag-1* flowers, which reveal other aspects of *AG* function. First, we observed inappropriate divisions in the L2. A number of *ag-1* flowers had a normal three-layered structure in the regions in which organs had not yet initiated. In approximately half (42/86) of the *ag-1* flowers examined, however, L2 cells divided periclinally into the L3, contributing to an abnormal extent to the receptacle of the main flower or that of the internal flowers. These aberrant divisions occurred in the center of the flower as early as stage 3 (Fig. 6F,G,H), but were more commonly observed in the center of the internal flowers. We have never observed this phenomenon in either wild-type or *ap3-3* flowers. The extent of the L2 invasion was variable, and it was usually limited to only part of the flower (Fig. 6I). Occasionally, L2-derived cells would completely replace the L3 from the growing point (Fig. 6J). The invasion was always inwards. We have never detected the L2 invading the L1, nor have we observed aberrant divisions in the L1. From these data it appears that normal *AG* function is required to prevent periclinal divisions in the L2 prior to organogenesis.

All *ag-1* mutant flowers observed also displayed, to variable extents, another phenotype. This phenotype consisted of an invasion of the L3 into the base of the organs. In general, the L3 contribution was one to three cells wide and extended distally about one third of the organ length (Fig. 6E). The invasion appeared to occur after the organs have initiated, by a division of an L3 cell into the organ (Fig. 6K). We observed this *ag-1* phenotype not only in all the organs of the internal flowers but also in the third and, most strikingly, the second whorl of the main flower (Fig. 6L). We have never detected an L3 contribution to the petals in wild-type flowers. These experiments suggest that normal *AG* function is required for appropriate cell divisions in the L3 after the initiation of the petal primordia. Since *AG* is not expressed in the second whorl (Drews et al., 1991), this is likely to be a non-autonomous effect.

DISCUSSION

A fate map of the *Arabidopsis* flower

The clonal sectors characterized in our study demonstrate that there is a very regular contribution of the meristematic cell layers to the *Arabidopsis* flower (Fig. 7). This fate map differs in a number of respects from those of other species; for instance, in *Arabidopsis* the perianth organs are composed almost exclusively of L1 and L2 cells. These species-specific variations presumably reflect differences in the developmental programs that give rise to the floral organs in each species. In addition, our results show that the formation of organs and particular tissue types relies on coordination of patterns of cell division and cell proliferation between different cell layers.

Plasticity in the development of the *Arabidopsis* flower

One important issue is whether layer contributions are identical in all the flowers of a plant or whether there is certain degree of flexibility. Our results show that the answer varies according to the layer and the tissue under consideration, with more plasticity in the L2 and L3 than in the L1. Layer invasions are more frequent in plants where a clone of cells has some growth disadvantage (Stewart et al., 1974) or in plants that are chimeras of different species (Marcotrigiano and Bernatzky, 1995). This is a critical consideration when performing mosaic analyses, in which clones of cells have different phenotypes and, potentially, different growth dynamics. For instance, Sieburth et al. (1998) in their mosaic analyses of *AG* observed a number of events that they interpreted as wild-type L1 or L3 invading an *ag* L2 in the internal flowers. We have not observed those types of invasions in flowers in which all cells are *ag*, but we did see L2 cells invading the L3. It is possible that in their study the wild-type and *ag* layers have different growth kinetics, resulting in the consequent invasion of *ag* tissue by wild-type cells. These results highlight the fact that the wild-type fate map may not accurately reflect layer contributions in the mutant. Therefore, a detailed analysis of the contributions of the cell layers in a particular mutant background is a necessary prerequisite for understanding the potential effects of cell proliferation conferred by the mutation, and the possible deviations from the wild-type fate map seen in mosaic plants.

The flexibility in the development of normal flowers and the existence of layer invasion events which do not affect the outcome of the developmental process reaffirms the idea that the final fate of plant cells is not dependent on lineage but on position. However, there is an apparent regulation in the orientation of the cell divisions during normal organ ontogeny, with some tissues (e.g. epidermis) displaying a tighter control than others (e.g. carpel wall mesophyll). There must be genetic programs that control the patterns of cell divisions in a layer specific manner, and those programs may differ according to the tissue and the developmental stage.

Two stages in the control of cell divisions in the FM

The results that we obtained for the clonal analyses of the homeotic mutants *ap3-3* and *ag-1*, in conjunction with other results reported in the literature, suggest that the control of cell proliferation in the flower can be divided in two stages. Until floral stage 6 the regulation of cell divisions is dependent on the position of the cells in the FM, but later the patterns of cell proliferation are dictated by the identity of the developing organs.

The early stage

The characteristic patterns of cell divisions at the inception of the organ primordia in each whorl are unaffected by mutations involving changes in the identity of the mature organs (our data; Bossinger and Smyth, 1996; Crone and Lord, 1994; Hill and Lord, 1989). Even though the organ identity genes are expressed as early as stage 3, demarcating the four whorls, the phenotypic defects in the transformed organs in flowers mutant for these genes are not apparent until after the organ primordia have appeared (stages 4 to 6) (our data; Bowman et al., 1989; Jack et al., 1992). Temperature shift experiments have suggested that the phenocritical phase for homeotic gene action is after stage 4 (*APETALA2*) or stage 5 (*AP3*) (Bowman et al.,

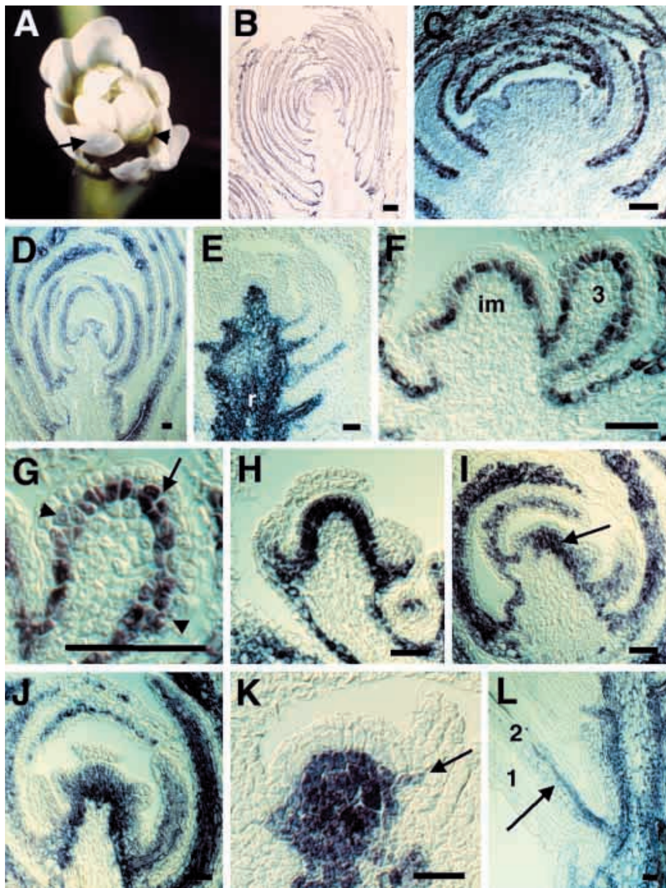


Fig. 6. Clonal analysis of the *ag-1* mutant. (A) *ag-1* flower. The third whorl is transformed into petals (arrow), the fourth into sepaloid organs (arrowhead) and more whorls are present in the center. (B-L) Longitudinal sections of *ag-1* flowers probed with anti-GUS antibody. (B,C) L1-labeled flower. The L1 only contributes to the epidermis. (D) L2-labeled flower. The L2 contributes to the mesophyll of the organs. (E) L3-labeled flower. The L3 contributes to the receptacle (r), and also to some of the mesophyll in the basal part of some organs. (F) Inflorescence meristem (im) and a stage 3 flower (3), with the L2 labeled. The inflorescence meristem appears wild type. (G) Detail of the stage 3 flower in F, showing aberrant L2 divisions in the center of the flower (arrows), and the normal L2 periclinal divisions that give rise to the sepal primordia (arrowheads). (H) L2-labeled stage 5 flower. The L2 has divided into the L3, displacing its top-most layer. (I,J) L2-labeled internal flowers. In I the L2 has invaded the L3 on one side of the meristem (arrow), while in J it has displaced the L3 completely from the apex. (K) L3-labeled internal flower showing the L3 dividing periclinally into the organ primordium (arrow). (L) An L3 (labeled) invasion into a second whorl (2) petal (arrow). Scale bars, 50 µm.

1989), and our analyses demonstrate that deviations from the wild-type pattern in homeotically transformed organs are not apparent until after stage 6. Together these results suggest that the initial specification of whorl-specific cell division patterns and layer contributions does not depend on the action of the floral homeotic genes.

What are the genes that regulate the initial patterns of cell divisions in the FM? Some of the processes taking place in the early FM are common to all shoot meristems. These include regulation of meristem size, the maintenance of histogenic layers and the distinction between a central zone of undifferentiated cells and a peripheral zone where organs start to differentiate (Steeves and Sussex, 1989). In the FM, the general meristem function genes (reviewed by Meyerowitz, 1997) may act together with the products of floral meristem identity genes like *LEAFY* (Parcy et al., 1998). The concerted actions of these groups of genes may be sufficient to establish both the radial pattern of homeotic gene expression, as well as to specify the initial patterns of cell divisions within each whorl. While none of these genes have yet been shown to have a specific effect on the proliferation of cells in particular histogenic layers, some of them (*CLAVATA1*, Clark et al., 1997; *WUSCHEL*, Mayer et al., 1998) have layer-specific expression patterns, and are possibly involved in coordinating proliferation of the different cell layers.

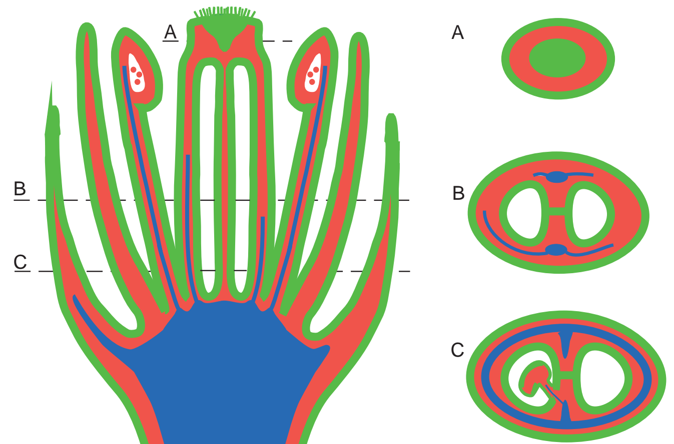


Fig. 7. Schematic representation of the contributions of the meristematic layers to the wild-type *Arabidopsis* flower. A, B and C are transverse sections of the pistil at the level of (A) the style, (B) the middle of the ovary, (C) the base of the ovary. L1, green; L2, red; L3, blue.

The late stage

By stage 7 we observe alterations in the layer contributions to the growing organ primordia, associated with the homeotic transformations caused by *ap3-3* and *ag-1* mutations. In both cases, the contribution of the derivatives of the meristematic layers corresponds to the new identity of the transformed organ, and not to its position in the flower. The data suggest that the organ identity genes specify identity only after the organ primordia have arisen. The organ identity genes can then be formally considered at the top of the genetic pathway that regulates patterns of cell divisions during organogenesis. Part of this hierarchy must entail tissue- and layer-specific regulation of cell divisions and growth to determine the final size and shape of the organs.

A role for *AGAMOUS* in meristem integrity

In addition to its role in regulating organ-specific patterns of cell division, we have shown that *AG* function is required for maintaining two different aspects of cell division patterns in the developing flower. First, as early as stage 3, we have observed inappropriate orientations of divisions in the L2 in *ag-1* mutant plants. Second, *ag-1* mutant plants also show an

excessive contribution of the L3 to many of the organs, including organs of the second whorl. Since *AG* is only expressed in the third and fourth whorls (Drews et al., 1991), this appears to be a non-autonomous effect. A non-autonomous role for *AG* in interlayer interactions has been proposed previously (Sieburth et al., 1998), but our results suggest that *AG* is also required for intercellular communication between different whorls, and for the maintenance of some aspects of meristem integrity. It could be argued that the effects seen are just a consequence of the increased proliferation associated with the indeterminate phenotype. However, we do not see a general breakdown in the orientation of cell divisions in the FM; rather we see very specific defects in these patterns. In addition, the fact that the layered structure is maintained in internal flowers in *ag-1* mutants also supports the idea that disruption of *AG* function does not lead to a general disorganization of cell division patterns. These observations suggest that the *AG* gene product may participate in regulating genes involved in specifying the patterns of cell divisions in different layers within the FM. Clonal analyses of mutants involved in meristem function should help to address this model.

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