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LEUNIG regulates *AGAMOUS* expression in *Arabidopsis* flowers

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SUMMARY

LEUNIG **was identified in a genetic screen designed to isolate second-site enhancer mutations of the floral homeotic mutant** ***apetala2-1*****.** ***leunig*** **mutations not only enhance** ***apetala2*****, but by themselves cause a similar but less-pronounced homeotic transformation than** ***apetala2*** **mutations.** ***leunig*** **flowers have sepals that are transformed toward stamens and carpels, and petals that are either staminoid or absent. In situ hybridization experiments with** ***leunig*** **mutants revealed altered expression pattern of the floral homeotic genes** ***APETALA1, APETALA3, PISTILLATA,*** **and** ***AGAMOUS*****. Double mutants of** ***leunig*** **and** ***agamous*** **exhibited a phenotype similar to** ***agamous*** **single mutants, indicating that** ***agamous*** **is epistatic to** ***leunig*****. Our analysis suggests that a key role of** ***LEUNIG*** **is to negatively regulate** ***AGAMOUS*** **expression in the first two whorls of the** ***Arabidopsis*** **flower.**

Key words: *Arabidopsis*, floral homeotic mutants, *leunig*, cadastral gene

INTRODUCTION

A fundamental question in plant development is that of how a multipotential cell becomes committed to a specific fate. Using *Arabidopsis* flower development as our model system, we have begun to understand how a group of undifferentiated cells in a floral meristem develop into a complex floral structure with four types of floral organs and many different cell types. Such a complex developmental process employs many regulatory genes with functions analogous to those involved in animal development. Floral homeotic genes (Bowman et al., 1989; Weigel and Meyerowitz, 1994), which are required to specify four types of floral organ identities, are similar to the homeotic selector genes that specify segment identity in flies (Lewis, 1978; Akam, 1987; Ingham, 1988). Genes that set the boundaries of floral homeotic gene function are analogous to the gap genes of flies (Reinitz and Levin, 1990), and are referred to as cadastral genes (Bowman et al., 1992; Weigel and Meyerowitz, 1994). This paper reports the identification and characterization of a novel cadastral gene, *LEUNIG* (*LUG*), in *Arabidopsis* flower development.

Arabidopsis thaliana flowers consist of four whorls of organs: 4 sepals, 4 petals, 6 stamens and 2 fused carpels arranged from the outermost (whorl 1) to the innermost (whorl 4) (Fig. 1A, B). A model has been established to account for how four different floral organ types are specified by region-specific activities of three classes (A, B, C) of floral homeotic genes (reviewed by Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). Class A genes are active in whorls 1 and 2 and are required for sepal and petal development. Class C genes act in whorls 3 and 4 and are required to specify stamen and carpel development. Class B genes function in whorls 2 and 3. In combination with class A genes, they specify petal development in whorl 2 and, along with the C genes, stamen development in whorl 3.

In *Arabidopsis*, described class A genes include *APETALA1* (*AP1)* and *APETALA2* (*AP2)*, class B genes are *APETALA3* (*AP3)* and *PISTILLATA (PI)*, and the known class C gene is *AGAMOUS (AG)*. All of these genes have been cloned (Yanofsky et al., 1990; Jack et al., 1992; Mandel et al., 1992; Goto and Meyerowitz, 1994, Jofuku et al., 1994). AP1, AG, AP3, and PI proteins all contain a conserved protein domain, the MADS domain, which is found in transcription factors in organisms ranging from yeast (MCM1, Passmore et al., 1988) to human (SRF, Norman et al., 1988). With gene-specific probes, the expression pattern of these four floral homeotic genes has been analyzed using in situ hybridization. Their RNA distribution largely coincides with the domain of their function. One class A gene, *AP1*, is expressed in whorls 1 and 2 in stage 3 and older flowers, although it is initially expressed in the entire floral primordium (Gustafson-Brown et al., 1994); the class B genes *AP3* and *PI* are largely expressed in whorls 2 and 3, although *PI* is initially expressed in whorls 2, 3 and 4 (Jack et al., 1992; Goto and Meyerowitz, 1994); the C class gene *AG* is expressed in whorls 3 and 4 (Drews et al., 1991).

At the molecular level, *AP2* is unique among these genes in that it does not encode a MADS box but rather a novel, putatively nuclear protein with two 68-amino acid repeat motifs (Jofuku et al., 1994). Despite its domain-specific function in whorls 1 and 2 (Kunst et al., 1989; Bowman et al., 1991), *AP2* RNA is detected in all four whorls of a flower as well as in vegetative tissues (Jofuku et al., 1994). Thus the domain-specific function of *AP2* may be conferred by domain-specific translational or post-translational controls, or by interaction with other domain-specific factor(s).

How is the domain of the A, B and C activities established? In *Arabidopsis*, meristem identity genes *LFY* and *AP1* initiate floral development by activating floral homeotic gene expression in the floral meristem (Weigel et al., 1992; Weigel

and Meyerowitz, 1993; Bowman et al., 1993) . The cadastral genes act next to define the boundaries of homeotic gene expression and function. For example, *SUPERMAN* (*SUP*) in *Arabidopsis* acts to prevent B class genes from functioning in whorl 4, and is therefore a cadastral gene (Bowman et al., 1992). The correct temporal and spatial pattern of B class gene expression and function is therefore controlled by the combined action of positive regulators *LFY* and *AP1* and the negative regulator *SUP*.

The class A gene *AP2* and the class C gene *AG* are also cadastral genes because they are involved in establishing the boundary between A and C activities. These two genes negatively regulate each other, and as a result, the A function is restricted to whorls 1 and 2, and the C function is restricted to whorls 3 and 4 (Bowman et al., 1991). In *ap2* loss-of-function mutants, *AG* activity expands into whorls 1 and 2, causing the formation of carpels in whorl 1 and stamens in whorl 2. In *ag* loss-of-function mutants, *AP1* and *AP2* organ identity functions expand into whorls 3 and 4, resulting in the conversion of stamens to petals, and carpels to sepals. This cadastral activity of *AP2* and *AG* is also revealed at the molecular level by in situ RNA hybridization. In *ag* mutants, *AP1* RNA expands to all whorls instead of being present only in whorls 1 and 2 (Gustafson-Brown et al., 1994); in *ap2-2* mutants, *AG* RNA is present in all whorls instead of only in whorls 3 and 4 (Drews et al., 1991). Since *AP2* RNA is distributed in all four whorls, the spatially restricted cadastral activity of *AP2* must depend on additional domain-specific cadastral factor (s) for *AG* repression. *AP1* is not such a factor because loss of *AP1* does not result in ectopic *AG* RNA expression (Gustafson-Brown et al., 1994).

To identify additional genes involved in *AG* regulation, we undertook a genetic screen for second-site enhancer or suppressor mutations of a weak *ap2* allele, *ap2-1* (Bowman et al., 1989). In *ap2-1*, whorl 1 organs are leaves instead of sepals; whorl 2 petals are staminoid; and whorl 3 and 4 organs are normal (Fig. 1C; Bowman et al. 1989, 1991). The *ap2-1* phenotype is very different from that of strong *ap2* mutants (Fig. 1D), and this difference results from intact or partially intact cadastral activity still present in the *ap2-1* mutants (Drews et al., 1991; Bowman et al., 1991). In this screen, we isolated two mutations that define a second-site enhancer of *ap2-1*, named *LEUNIG (LUG)*. Although our *lug* mutations were found to be allelic to a mutation in strain *Fl-89*, previously thought primarily to affect carpel fusion (Komaki et al., 1988), the enhancer screen has revealed an additional role of *LUG* in floral organ specification. The analysis of *lug* mutants reported here indicates that *LUG* is a cadastral gene involved in A and C boundary establishment during *Arabidopsis* flower development.

MATERIALS AND METHODS

Genetics

ap2-1 homozygous seeds were washed in 0.1% Tween-20 for 15 minutes, subsequently mutagenized with 0.1% EMS (ethylmethane sulfonate) for 8 hours, then washed with sterile water several times for a total of 4.5 hours, and sown on soil mix. 5,000 M1 plants germinated and gave rise to M2 seeds. The use of such a low concentration of EMS is based on our observation that *ap2-1* seeds are more susceptible to EMS than wild-type seeds. 42% of the mutagenized M1 plants possessed siliques segregating one-quarter embryonic lethals. M2 seeds were collected as families (10 M1 plants per family). Approximately 120 M2 plants were screened per family. 334 families out of the 500 families were screened.

The isolated enhancer mutants were crossed into wild-type Landsberg *erecta*: (L-er) plants, and all F_1 progeny were wild-type, indicating that both the *ap2-1* and the enhancer mutations are recessive to wild-type. For extragenic enhancers, the F_2 progeny segregated both *ap2-1* and the enhancer mutations. For intragenic *ap2* enhancers, *ap2-1* plants were not recovered in the F_2 generation.

Two independent *lug* mutations were obtained from screening progeny of 3340 M1 plants. The frequency of mutations in the *LUG* gene was thus roughly 1 in 1670 M1 plants. This is probably an underestimate, because at least one more *leunig*-like mutant was identified and then lost due to poor fertility.

The map location of *lug* was determined by its linkage to *ap2* and *ag* on chromosome 4. According to the frequency of recombination between *ap2* and *lug* and between *ag* and *lug*, *lug* is situated between *ap2* and *ag* 16 map units above *ap2* and 14 map units below *ag* (data not shown).

ap2-9 lug-1 double mutants were constructed by crossing homozygous *ap2-9* (L-er) pollen to *lug-1* (L-er) carpel to generate F_1 *trans*-heterozygotes. Twenty *lug-1* single mutant plants in the F_2 generation were selfed and planted as individual families. 2 such *lug-1* plants segregated plants with three different phenotypes: (A) *lug-1* single mutant phenotype; (B) *ap2-9 lug-1* double mutant phenotype; (C) an intermediate phenotype between class A and B. To confirm that the class C represents *lug-1* plants heterozygous for *ap2-9*, seeds from individual plants in class A and C were collected (class B is completely sterile). Class C plants all segregated *ap2-9 lug-1* double mutants; whereas class A did not segregate any *ap2-9 lug-1*.

Scanning electron microscopy

Samples were collected, fixed, coated, and photographed as described previously (Bowman et al., 1989, 1991).

In situ hybridization

For radioactive in situ hybridization, all flowers were collected, fixed, embedded, sectioned, and hybridized as described previously (Drews et al., 1991).

For non-radioactive in situ hybridization (Fig. 4), the fixation, embedding and sectioning steps were essentially the same as for radioactive hybridizations except that the fixation step was shorter (1 hour) and the time between infiltration steps was minimized. The probes were synthesized using the DIG (digoxigenin) RNA labeling kit (Boehringer Mannheim Biochemical) according to the manufacturer's instructions. Slide treatment before hybridization was similar to that of radioactive in situ hybridization. Subsequent hybridization, wash, signal detection steps were modifications of Langdale et al. (1987).

Antisense probes were made from pCIT 565 for *AG* (Yanofsky et al., 1990), pAM 128 for *AP1* (Mandel et al., 1992), pD793 for *AP3* (Jack et al., 1992), and pcPINX for *PI* (Goto and Meyerowitz, 1994).

Image processing

Negatives and slides were scanned and digitized using a Nikon Coolscan. Brightness and contrast were adjusted using Adobe Photoshop 3.0, and for the in situ double exposures, the color balance was similarly adjusted. Final figures were printed using a Kodak XLS 8300 Digital Printer.

Plant growth

Seeds were sown in a 1:1:1 mixture of perlite:vermiculite:soil, incubated at 4°C for 4 days, and then placed under lights. Biological larvicide Gnatrol (Abbott Laboratory) was added to the water used to moisten the soil mixture before sowing. Unless otherwise noted, all plants were grown under 600 ft-candles of constant cool white fluorescent light at 22-24°C. Plants were fertilized at about 10 days after germination with Plantex all purpose fertilizer.

RESULTS

Identifying *leunig*

About 5,000 M1 *ap2-1* seeds germinated after the EMS (ethylmethane sulfonate) treatment, and about 27,400 M2 *ap2-1* plants, representing 3340 M1 plants, were screened to identify mutations that cause either a suppressed phenotype resembling wild-type, or an enhanced phenotype resembling strong *ap2* mutants. Thirteen putative enhancer mutations with phenotypes resembling strong *ap2* mutants were identified (Fig.

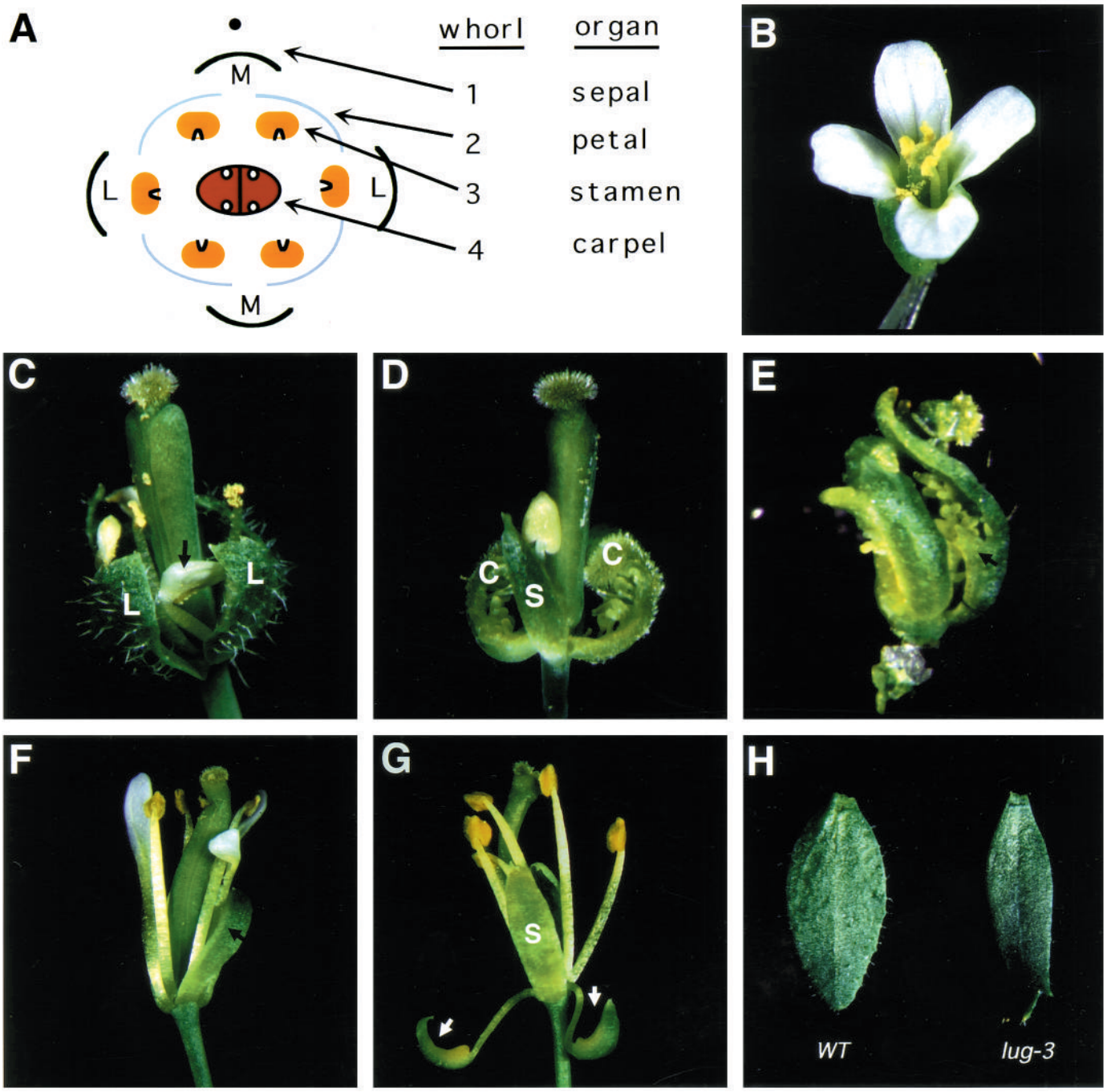

Fig. 1. Structure and phenotypes of wild-type and mutant *Arabidopsis* flowers. (A) A diagram of a wild-type *Arabidopsis* flower. The black dot indicates the inflorescence meristem (IM). Abbreviations are: M, medial position with respect to IM; L, lateral position with respect to IM. The medial adaxial sepal is adjacent to IM, and the medial abaxial sepal is opposite to IM. (B) A wild-type *Arabidopsis* flower. (C) An *ap2-1* flower with leaves (L) in whorl 1, staminoid petals (arrow) in whorl 2, and normal stamens and carpels in whorls 3 and 4. (D) An *ap2-2* flower with medial carpels (C) and lateral sepals (S) in whorl 1. Whorl 2 and 3 organs are reduced to a single stamen. (E) An *ap2-1 lug-1* flower with medial whorl 1 carpels (arrow) and whorl 4 unfused carpels. Lateral whorl 1 organs, and whorl 2 and 3 organs are absent. (F) A basal *lug-1* flower with narrow floral organs. The thickening of the sepal edge (black arrow) indicates slight carpelloidy. (G) An apical *lug-1* flower. Medial whorl 1 sepals are staminoid (arrows), lateral whorl 1 sepals (S) are normal, and whorl 2 petals are absent. (H) Cauline leaves of wild-type (WT, L-er ecotype) and *lug-3*.

1E); however, no suppressor mutations were isolated. Segregation tests established that only two of the 13 enhancer mutations are second-site mutations and the remaining 11 appear to be intragenic *ap2* mutations (see Materials and Methods).

The two second-site enhancer mutations are recessive, exhibit similar phenotypes, and fail to complement each other, thus defining a single genetic locus. This locus was mapped to chromosome 4 (see Materials and Methods). The two mutations were subsequently shown to be allelic to two mutations previously isolated by D.R. Smyth in our laboratory, called *leunig* (*lug*) and to a mutation in a strain named *Fl-89* (Komaki et al., 1988). Several new *lug* alleles were later obtained (Table 1).

Morphological characterization of *lug* mutants

We have analyzed a total of 10 alleles of *lug* (Table 1), all of which cause similar recessive phenotypes. First of all, *lug* mutants are characterized by narrow leaves and floral organs (Fig. 1F-H). Secondly, homeotic transformation in floral organ identity is frequently observed in whorls 1 and 2 (see below). Thirdly, reduction in organ number occurs frequently in whorls 2 and 3 (Fig. 1F,G). Finally, whorl 4 carpels fail to fuse properly (below). The defects in floral organ identity and floral organ number are more severe in flowers arising later in an inflorescence shoot, that is in a more apical position (Fig. 1 compare F and G). Thus it is important to distinguish "early-arising" (basal) flowers (Fig. 1F) from "late-arising" (apical) flowers (Fig. 1G) on the same inflorescence. When we examined young inflorescences of *lug-1* by scanning electron microscopy (SEM), the flower primordia appear normal (Fig. 2, compare A to F), however, the floral organ primordia are narrow in *lug*. In old inflorescences of *lug-1* (Fig. 2G), flower primordia are abnormal in shape. In more apical flowers, the size of the center dome interior to whorl 1 organs is much reduced (Fig. 2L, Q), thus insufficient central primordial cells may be responsible for the reduced number of floral organs in more apical *lug* flowers. Specific effects of *lug* on each whorl are described in detail below.

Table 1. Sources of *lug* alleles

Allele*	Isolation no.	Mutagen	Effect†	Origin
1	38	EMS	Intermediate	Present study
2	70	EMS	Strong	Present study
3	68-2	EMS	Strong	G. Fox and T. Jack
4[a]	60-2	EMS	Strong	G. Fox and T. Jack
5	1-4	EMS	Strong	J. Levin
6	2B11	EMS	Intermediate	D. Weigel
7	Morph 3	EMS	ND	S. Jacobsen
8	S24	EMS	Weak	D. Smyth
9	S42	EMS	Weak	D. Smyth
10	Fl-89	EMS	Weak	K. Okada‡

*All alleles were induced in Lansberg *erecta* background.

†"weak", "intermediate", and "strong" alleles are classified according to how soon an inflorescence gives rise to flowers that exhibit reduced number of petals and carpelloid/staminoid sepals. Stronger mutations exhibit defects sooner than weaker alleles. In flowers at more apical positions, different alleles exhibit similar phenotypes.

‡Komaki et al., 1988.

4[a]: this allele exhibits mosaic whorl 1 organs at a high frequency.

Whorl 1 effects

Partial homeotic transformation is frequently observed in whorl 1. Whorl 1 sepals are frequently petaloid, staminoid or carpelloid (Fig. 2, compare B-D with H-J, M and N; Table 2). In basal flowers, whorl 1 medial positions can be occupied by sepals with petaloid tissue at their margins (Fig. 2H). In apical flowers, whorl 1 medial organs can be staminoid or carpelloid (Fig. 2I,J,M), or stamen/carpel mosaics with ovules developing along the margins of the mosaic organs (Table 2; data not shown). The medial adaxial sepal is more transformed than the medial abaxial sepal. Lateral whorl 1 organs are less affected

Fig. 2. Scanning electron microscopic (SEM) pictures of wild-type and *lug* single mutants. Bars equal 10 μm in A-D, F-L, O-R; bars equal 100 μm in E, M, N, R, S. Numbers indicate the stages of respective flowers according to Smyth et al. (1990). Abbreviations are: IM, inflorescence meristem; S, sepal; P, petal; st, stamen; C, carpel or carpelloid; st/S, staminoid sepal; O, ovule; H, horn-like; F, filament; sb, stigmatic bundle. (A) A wild-type inflorescence. (B) Close-up of wild-type sepal tissues. Note the sepal-specific long cells and the stoma (arrow). (C) Close-up of wild-type petal cells at stage 11. (D) Close-up of mature wild-type stamen anther cells. (E) Top view of a fused wild-type carpel at stage 11. (F) A young *lug-1* inflorescence which is similar to wild-type with the exception of narrow floral organ primordia. (G) An old *lug-1* inflorescence. Note the abnormal shape of each floral primordium. In the stage 5 flower, the asterisk (*) indicates a whorl 2 organ, which is located abnormally inbetween the whorl 1 organs and may fuse with the whorl 1 organs. The arrow points to the absence of stamen and petal primordia in the stage 5 flower (compared with K). (H) Close-up of a *lug-4* whorl 1 organ mosaic for petal (P) and sepal (S) tissues. (I) Close-up of a *lug-2* whorl 1 organ mosaic for stamen (st) and sepal (S) tissues. (J) Close-up of a *lug-2* whorl 1 organ mosaic for carpel and sepal (S) tissues. Note the two ovules (O) developing from the margin. (K) A stage 5 wild-type flower. Two whorl 1 sepals have been removed to reveal the stamen (st) and petal (arrow) primordia. (L) An apical *lug-2* stage 4 flower. Note the much reduced central dome (compared with the stage 4 flower in A). (M) A *lug-1* flower dissected to reveal the similarity of a carpelloid whorl 1 organ to the whorl 4 carpel (C). Note the presence of carpel-specific characters in the whorl 1 organ: stigmatic tissue (black arrow) and the ovules (white arrows). (N) A *lug-2* flower with whorl 1 medial carpels, whorl 1 lateral staminoid sepals (st/S). Ovules (black arrows) are visible near the base of whorl 1 carpels. The narrow and elongated tips (white arrows) of whorl 1 carpels resemble the horn-like protrusions of *lug* carpels (see T). (O) An unfused carpel of *lug-2*. One of the carpels is filament-like (F). The ovules developing from the placenta appear normal at this stage. (P) A dissected stage 7 wild-type flower. All sepals have been removed showing petal (P) stamen (st), and carpel (C) primordia. (Q) An apical *lug-2* stage 6 flower. The two medial carpelloid sepals (S) are dissected away, so are the two lateral filamentous sepals (arrows). Note the much reduced central primordium and the absence of stamen and petal primordia (compared with P). Fusion may occur between the two medial whorl 1 organs and the inner organs (most likely, with the whorl 4 carpels). (R) A whorl 2 mosaic organ in *lug-4* with petal (P) blade on top and a stamen filament (F) at the base. (S) A *lug-2* flower with an abnormally developing stamen. Note the asynchronous development of the two anther thecae (arrows). This stamen is flanked by a filament (F) and an unfused whorl 4 carpel (C). (T) An unfused gynoecium of *lug-3* showing the two horn-like (H) protrusions and the two unfused stigmatic bundles (sb). The two horns are extensions of the carpel valves, and the stigmatic bundles grow out of the septum region.

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Table 2. Comparison of organ types in *lug-1* mutant and wild-type flowers

Whorl	Position (organ number)	Organ identity	Wild type* %	*lug-1* 1-10 (29)† %	*lug-1* 11-20 (25)† %	*lug-1* 21-30 (21)† %
1	Medial	Sepals	100	60	10	0
	(2)	Staminoid	0	17	52	57
		Petaloid	0	10	8	0
		St/Ca‡	0	5	14	36
		St/Pet‡	0	5	0	0
		Pet/Ca‡	0	0	8	0
		Carpelloid	0	1	6	7
		Absent	0	0	2	0
	Lateral	Sepals	100	96	48	17
	(2)	Petaloid	0	3	52	38
		Staminoid	0	0	0	2
		Caplloid	0	0	0	23
		Others	0	0	0	20
2	(4)	Petals	100	47	15	6
		Staminoid	0	9	5	0
		Filaments	0	2	2	0
		Absent	0	42	78	94
3	(6)	Stamens	99	66	54	49
		Filaments	0	0.5	0	2
		Absent	1	33	46	49
4	(2)	<2 Carpels	0	17	12	14
		2 Carpels	100	76	80	86
		>2 Carpels	0	7	8	0

*From Smyth et al., 1990.

†The numbers 1-10, 11-20, and 21-30 indicate the positions of the flowers in an inflorescence with 1 = most basal position; the numbers in the parentheses indicate the number of flowers scored.

‡St/Ca:staminoid carpel; St/Pet: staminoid petal; Pet/Ca: petaloid carpel. Also see Bowmen et al. (1991).

by *lug* mutations than medial ones, as is also true of *ap2-2* mutants (Fig. 1D; Bowman et al., 1991). The lateral whorl 1 organs can develop into sepals, petaloid sepals, staminoid sepals, carpelloid sepals or filaments (Figs 1G, 2N; Table 2). Frequently, carpelloid sepals of *lug* mutants lack stigmatic tissues, are much elongated, and exhibit horn-like protrusions at the tips characteristic of whorl 4 *lug* carpels (Fig. 2N,T).

Whorl 2 effects

Homeotic transformation is observed in whorl 2 petals with stamen characteristics: stamen-like filaments at the base (Fig. 2R) or anther tissue at the top (data not shown). In addition, *lug* flowers have a reduced number of petals in whorl 2, especially in apical flowers (Table 2; Fig. 1F,G). Loss of petals likely results from the fact that the central dome is much reduced in size (Fig. 2L,Q). Thus, insufficiency of central primordial cells may be responsible for the reduction in floral organ initiation. Fusion between whorl 2 organs and whorl 1 organs, although rarely observed, may also account for some of the reduced petal number (see Fig. 2G and legend).

Whorl 3 effects

In *lug* mutants, stamens are reduced in number (Table 2), presumably due to the reduction of stamen primordium initiation (Fig. 2; compare G, L, Q to A (stage 4), K (stage 5), and P (stage 7)). Occasionally, anthers senesce prematurely or are malformed (Fig. 2S).

Whorl 4 effects

The number of whorl 4 carpels in *lug* flowers varies from 1.5 (with one fully developed and one half-developed carpel) to 4 (Table 2). The reduction in carpel number results from early abortion, filamentous growth or retarded development of one or both of the carpels (Fig. 2O). Increase in carpel number, however, is attributed to additional filamentous organs fused at the base of carpels at medial positions and thus may merely reflect an artificial assignment.

In almost every *lug* flower, the carpels fail to fuse properly (compare Fig. 2T and E). We frequently observed horn-like protrusions at the tip of each carpel valve as well as two stigmatic bundles topping two septum tissues (Fig. 2T; Komaki et al., 1988). Wild-type pollen does not rescue low female fertility of *lug* mutants, indicating possible defects in ovule development and/or septum transduction for pollen tube growth.

lug mutations cause ectopic B and C homeotic gene expression

According to the ABC model (reviewed by Weigel and Meyerowitz, 1994), the homeotic transformation observed in whorls 1 and 2 of *lug* flowers suggests that both C and B class genes are ectopically active. Since the RNA expression pattern of both C and B class genes correlates with their functions (Drews et al., 1991; Jack et al., 1992; Goto and Meyerowitz, 1994), we sought to examine the RNA expression pattern of *AG*, *AP3*, and *PI* in *lug* mutants by use of in situ hybridization.

Expression of the C class gene *AG*

Both the temporal and the spatial pattern of *AG* expression is altered in *lug* flowers (Fig. 3). In flowers of wild-type, *ap2-1* (Drews et al., 1991), and *ap1-1* (Fig. 3D; Gustafson-Brown et al., 1994), *AG* RNA is not detected in stage 1 and stage 2 floral primordia; a low level of *AG* RNA starts in the center of early

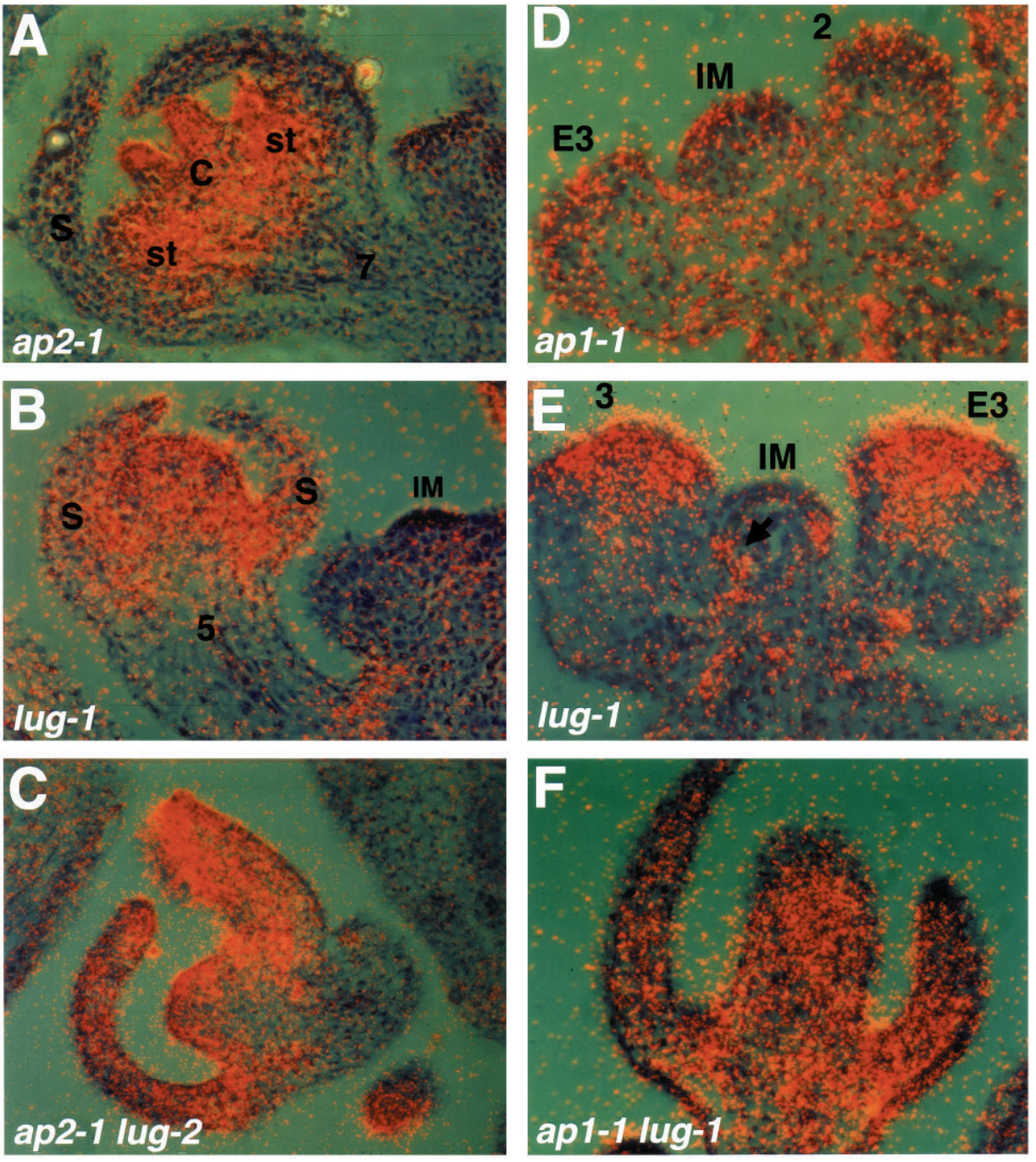


Fig. 3. *AGAMOUS* (*AG*) expression in single and double mutants. In situ hybridization of a radioactive (^{35}S) *AGAMOUS* antisense probe to 8 μm longitudinal sections of plant inflorescence apices. The flowers shown are at apical positions 10-20th. The tissues were stained blue with 0.1% toluidine blue. Photos were taken using bright-field and dark-field double exposures with a red filter during dark exposure. Red grains represent signal. Numbers indicate the stages of corresponding flowers according to Smyth et al. (1990). (A) *AG* expression in a stage 7 *ap2-1* flower. Similarly to wild-type (Drews et al., 1991), *AG* RNA is detected in developing stamens (st) and carpels (C), but is absent from sepals (S). Petals are still small and not visible in this section. (B) *AG* expression in a stage 5 *lug-1* flower. *AG* RNA is detected in both sepals (S) and the center dome that will give rise to whorls 2, 3 and 4. (C) *AG* expression in a stage 7 flower of genotype *ap2-1 lug-2* . *AG* RNA is detected in both sepals and in the whorl 4 carpels. Organs in whorls 2 and 3 are severely reduced in number and are absent in this section. (D) *AG* expression in *ap1-1*. Shown are the inflorescence meristem (IM), a late-stage 2 floral primordium (right), and an early stage 3 (E3) floral primordium (left). No *AG* expression is detected above the background. This AG expression is similar to wild-type and *ap2-1* (Drews et al., 1991). (E) Precocious *AG* expression in *lug-1*. Shown is an inflorescence meristem (IM), an early stage 3 (E3) floral primordium (right), and a stage 3 floral primordium (left). *AG* expression is detected in both of the floral primordia, but is absent from the IM. The early stage 3 flower (right) is at a similar developmental stage to the early stage 3 (E3) flower of *ap1-1* shown in D. These two early stage 3 flowers are distinct in the ability to express *AG*. Note the patches of *AG* RNA in the areas (arrow) of stem and IM. (F) *AG* expression in a stage 8 flower of genotype *ap1-1 lug-1*. *AG* is detected in all the existing whorls.

stage 3 floral primordia and, by mid stage 3, expands to encompass the region that later gives rise to stamens and carpels. However, in *lug* (examined alleles: *lug-1, lug-3,* and *lug-4*) flowers, *AG* RNA is detected starting at mid stage 2 (data not shown) and is abundant at early stage 3 (Fig. 3E). The amount of *AG* RNA detected in early stage 3 *lug* flowers is greater (Fig. 3E) than that detected in wild-type early stage 3 flowers (Drews et al., 1991). Similar precocious *AG* expression was also observed in strong *ap2-2* mutants (Drews et al., 1991), in *ap2-1 lug* (*ap2-1 lug-1* and *ap2-1 lug-2*) and *ap1-1 lug-1* double mutants (data not shown).

In wild-type, *ap2-1,* and *ap1-1* backgrounds, *AG* RNA expression is restricted to whorls 3 and 4 of stage 3 and older flowers (Fig. 3A; Drews et al., 1991; Gustafson-Brown et al., 1994). However, in *lug* single (*lug-1, lug-3,* and *lug-4*), *ap2-1 lug* (*ap2-1 lug-1* and *ap2-1 lug-2*) or *ap1-1 lug-1* double mutant flowers, *AG* RNA is detected in all existing whorls (Fig. 3B,C,F). This ectopic *AG* expression is partial in *lug* single mutants and is complete in *ap2-1 lug* or *ap1-1 lug-1* double mutants. In *lug-1,* 10 out of 17 medial whorl 1 organs express *AG* (58%). However, *AG* RNA is detected in all medial whorl 1 organs in *lug-1 ap2-1* (100% or 16/16).

The expression of B class genes *AP3* and *PI*

Both *AP3* and *PI* are ectopically expressed in whorl 1 of *lug* mutants. In wild-type, *AP3* RNA is detected in primordia of whorl 2 and 3 organs as well as at the base of whorl 1 organs (Jack et al., 1992; Weigel and Meyerowitz, 1993); *PI* RNA is first detected in regions of floral primordia that give rise to whorls 2, 3 and 4 (stages 3 and 4), and later is confined to the whorls 2 and 3 (Goto and Meyerowitz, 1994). However, both *AP3* RNA and *PI* RNA are detected in whorl 1 organs of *lug-1* mutants (Fig. 4). In *lug-1*, the frequency of ectopic *PI* gene expression is 59% (30/51 whorl 1 organs), and for *AP3* is 50% (11/22 whorl 1 organs). This is consistent with the incomplete transformation of whorl 1 sepals into staminoid or petaloid sepals.

ag is epistatic to *lug* with respect to floral organ identity

If staminoid whorl 2 and staminoid/carpelloid whorl 1 organs in *lug* flowers result from ectopic *AG* activity, eliminating *AG* activity in the *lug* background would result in normal petals in whorl 2 and normal sepals as well as petaloid sepals in whorl 1 (assuming that occasional ectopic B activity is still present in whorl 1). We constructed *lug-1 ag-1* double mutants and observed that the double mutant flowers develop sepals in whorl 1 and petals in whorl 2 with correct organ identity and organ number (Fig. 5C). The similarity of *lug-1 ag-1* flowers to *ag-1* flowers (Fig. 5 compare A and C) suggests that *ag* is epistatic to *lug* with respect to floral organ identity. This property of *LUG* is in sharp contrast to *AP2*, because *ap2-1 ag-1* double mutants do not have normal sepals and petals (Fig. 5B; Bowman et al., 1991).

Flowers of *lug-1 ag-1* still exhibit narrow leaves, sepals and petals (Fig. 5C), indicating an *AG*-independent role of *LUG* in controlling organ shape.

Whorl 1 organs of *lug-1 ag-1* flowers lack petaloid sepals (Fig. 5C). In *lug-1*, staminoid sepals occur at a frequency of 71% (10-20th flowers). These staminoid sepals would become petaloid sepals upon the removal of ectopic *AG* in *lug-1 ag-1*. However, only subtle petaloid margins were occasionally (16%) observed in whorl 1 organs (10-20th flowers) of the double mutants. Thus the ectopic B activity is reduced in the absence of ectopic *AG* activity.

lug enhances the defects of class A mutants, *ap2* and *ap1*

ap2

ap2-1 lug-1 double mutants exhibit more severe homeotic transformation in flowers than either single mutant. *ap2-1*, a weak *ap2* allele, develops leaves in whorl 1, staminoid petals in whorl 2 (reduced in number), and largely normal stamens and carpels in whorls 3 and 4 (Figs 1C, 6A). In contrast, *ap2-1 lug-1* double mutants develop filaments in lateral positions and carpels in medial positions in whorl 1; whorl 2 organs are completely absent, between 0 to 3 stamens are made in whorl 3; and whorl 4 carpels are unfused (Figs 1E, 6B). This is consistent with our earlier observation that *lug-1 ap2-1* double mutants misexpress *AG* at a higher frequency than either single mutant (see earlier section). Furthermore, we frequently observed two lateral filaments arising below the two lateral sepals on the pedicels of the double mutants (Fig. 6C). These lateral filaments were rarely observed in *lug* and *ap2* single mutants. Carpels in *lug-1 ap2-1* flowers are not fused and the plants are completely female-sterile. However, *lug ap2* double mutants do not exhibit an enhanced phenotype in floral organ shape and leaf shape.

Dominant interactions were observed between strong *ap2* and *lug*, though either mutation alone is recessive. *lug-1* plants heterozygous for *ap2-9* exhibit a floral phenotype more severe than *lug-1* (Fig. 7A,B, Materials and Methods). *lug-1/lug-1 ap2-9/+* flowers have carpelloid sepals in whorl 1 and have lost most or all of the whorl 2 petals, as in *lug-1 ap2-1* double mutants. However, *lug* mutations appear recessive in an *ap2-9/ap2-9* background. The dominant interaction between *lug-1/lug-1* and *ap2-9/+* suggests that the products of *AP2* and *LUG* may interact by direct contact, or by defining the same activity at a threshold level (see Discussion).

lug-1 ap2-9 homozygous flowers exhibit stronger defects than the strongest *ap2-2* single mutants (compare Figs 6E, 7D with 1D and refer to Bowman et al., 1991). Each floral primordium of *lug-1 ap2-9* double mutants is subtended by a bract-like organ at the abaxial position (Fig. 6F). A "bract" usually refers to a small leaf subtending flowers (Gifford and Foster, 1988) and is usually absent in *Arabidopsis*. However, bract-like organs are observed in several *Arabidopsis* mutants including *lfy* (Weigel et al., 1992; Huala and Sussex, 1992) and *ap1* (Irish and Sussex, 1990; Bowman et al., 1993). The floral primordium of *lug-1 ap2-9* develops into a single central gynoecium with or without two filamentous whorl 1 organs (Figs 6E, 7D). This stronger defect of *lug-1 ap2-9* double homozygotes suggests that *LUG* and *AP2* have overlapping but not completely redundant functions.

ap1

In *ap1-1* mutant flowers, whorl 1 sepals are transformed into leaves or bracts with axillary flowers developing in their axils. Whorl 2 organs are either absent or are staminoid petals, staminoid, or leaf-like. The whorl 3 and 4 organs are similar to wild type (Fig. 6G; Irish and Sussex, 1990; Bowman et al.,

1993). Thus *AP1* is required for sepal and petal identity and is a class A gene. *lug-1 ap1-1* mutants are similar to *lug-1 ap2-1* double mutants or strong *ap2* single mutants (Fig. 6 compare H with B, and I with C). Medial whorl 1 organs are converted to carpels; lateral whorl 1 organs are filamentous or aborted. Axillary flowers are absent as are floral organs in whorls 2 and 3. This enhanced phenotype in *lug-1 ap1-1* double mutants is consistent with our observation that *lug-1 ap1-1* flowers exhibit enhanced ectopic *AG* expression (see earlier section), which may suppress axillary flowers. Similarly to *lug-1 ap2-1* double mutants, *lug-1 ap1-1* double mutant plants do not exhibit an enhanced phenotype in vegetative tissues.

It has been shown that *AP1* RNA accumulation is negatively regulated by *AG*. (Gustafson-Brown et al., 1994). We tested whether ectopic *AG* activity in whorls 1 and 2 of *lug* or *lug ap2-1* mutants could repress *AP1* RNA accumulation, by the use of in situ hybridization. In wild-type or *ap2-1* (Fig. 8A; Gustafson-Brown et al., 1994), *AP1* RNA is first expressed in the entire floral primordium; by stage 3, *AP1* RNA is restricted to whorls 1 and 2 as a result of *AG* expression in the center of the flower. *AP1* RNA is also normally expressed in pedicels of flowers. In *lug* mutants (*lug-1, lug-3, lug-4*), *AP1* RNA is frequently detected in only one of the two whorl 1 organs in longitudinal sections (Fig. 8B). In *lug-1* single mutants, 50% (22/43) of whorl 1 organs fail to express *AP1*. This defect is more severe in an *ap2-1 lug-1* double mutant (Fig. 8C), in which 87% (26/30) of the whorl 1 organs fail to express *AP1*. Nonetheless, *AP1* RNA is still detected in pedicels of *lug* and *ap2-1 lug-1* flowers (Fig. 8C). In *ag-1 lug-1* flowers, *AP1* RNA is detected in all whorls (Fig. 8D), suggesting that the ectopic *AG* expression in *lug* is responsible for the absence of *AP1* RNA accumulation.

lug and B class double mutants have additive phenotypes

The strong *ap3* mutation, *ap3-3*, converts whorl 2 organs into sepals, and whorl 3 organs into carpels, which usually fuse with the central gynoecium (Fig. 7F; Jack et al., 1992). In addition, *ap3-3* mutations can result in loss of some organs in whorl 3 (Bowman et al., 1989; Jack et al., 1992). In *lug-3 ap3-3*, whorls 3 and 4 are similar to those of *ap3-3* in terms of organ identity, but possess a severely reduced number of whorl 3 carpels; whorl 2 organs are absent and whorl 1 consists of medial carpelloid sepals; lateral sepals are absent or filamentous (Fig. 7G). Evidently, the staminoid sepals found in *lug-3* flowers (Fig. 7E) are equivalent to the carpelloid sepals in the *lug-3 ap3-3* double mutants (Fig. 7G), indicating persistent presence of ectopic *AG* activity in the absence of ectopic B activity. Again, carpels of *lug-3 ap3-3* mutants exhibit a narrow organ shape and horn-like protrusion (Fig. 7G). Similar results were obtained with *lug-1 pi-1* double mutants (data not shown).

Since the simultaneous presence of ectopic *AP3* and *PI* is required for ectopic B activity, we crossed a *35S-AP3* transgene into *lug-1* to test whether *lug-1* causes ectopic *PI* activity. When introduced into wild-type plants, the transgene 35S-*AP3* ectopically directs expression of *AP3* RNA in all four whorls, thereby transforming carpels into stamens in whorl 4. Whorl 1 organs, however, remain sepals due to the absence of *PI* expression (Fig. 6J; Jack et al., 1994). *lug-1/lug-1 35S-AP3/+* flowers exhibit additive effects in whorls 2, 3 and 4: petals or staminoid petals in whorl 2, stamens in whorls 3 and 4. However, the medial whorl 1 organs are completely transformed into stamens (Fig. 6K,L), while the lateral organs remain sepals. Thus, *lug* results in ectopic *PI* activity in the medial whorl 1 organs, and the transgene enhances the homeotic transformation of these organs by providing abundant *AP3* activity.

sup and *lug* mutations are additive

sup mutant flowers develop extra stamens at the expense of the central gynoecium (Bowman et al., 1992; Schultz et al., 1992). Thus *SUP* is required to prevent B activity in whorl 4. *lug-1 sup-4* double mutants exhibit additive effects (Fig. 7H,I). Whorls 3 and 4 are *sup-4* like: stamens are formed at the expense of carpel tissues; the outer two whorls are *lug-1* like: carpelloid sepals and reduced number of petals. The number of stamens is reduced in the double mutant compared to *sup-4* alone.

Early termination of inflorescences in *lfy lug* double mutants

The strong *lfy* mutation, *lfy-6*, causes partial conversion of floral meristems to shoot meristems, and *lfy-6* inflorescences terminate with bracts, carpelloid bracts or a carpelloid mass (Weigel et al., 1992). *lug* mutations facilitate the termination of inflorescence shoots in *lfy-6*. *lug-3 lfy-6* inflorescences give rise to 3-4 cauline leaves (slightly fewer than *lfy-6* alone) with secondary shoots in their axils, and then 3-4 flowers with subtending bracts. Soon afterward, the inflorescence terminates with bracts or carpelloid bracts (Fig. 7L). This is in contrast to both *lug-3* and *lfy-6* single mutant inflorescences, which produce at least 25-30 flowers before termination (Fig. 7J,K). Double mutants between a weak *lfy* allele, *lfy-5* , and *lug-1* exhibit similar early termination (Fig. 6O). This phenotype is similar to the early termination observed in *lfy tfl* double mutants (Shannon and Meeks-Wagner, 1993; Schultz and Haughn, 1993).

In addition, *lug-1* appears to enhance the floral phenotype of the weak *lfy-5* mutants (Fig. 6 compare M with N). *lfy-5* and *lfy-6* single mutants have flowers of very different phenotypes (Figs 6M, 7K; Weigel et al., 1992); *lug-1* enhances the floral phenotype of *lfy-5*, and *lfy-5 lug-1* double mutant flowers are similar to those of *lfy-6* . Nevertheless, *lfy lug* double mutants have narrow leaves and floral organs (Figs 6N,O, 7L).

DISCUSSION

LUG is a class A cadastral gene

This study indicates that *LUG* is a negative regulator of *AG* (Fig. 9A). Both the organ identity transformation and the organ number reduction in *lug* mutants are mediated through ectopic *AG* expression**.** Unlike *AP2, LUG* is not required to specify sepal or petal identity, shown by the fact that *lug-1 ag-1* double mutants develop normal sepals and petals (narrow in shape). Thus, *LUG* is a cadastral gene whose main role in the determination of floral organ identity is to negatively regulate *AG*.

Class A function in whorls 1 and 2 can be divided into two

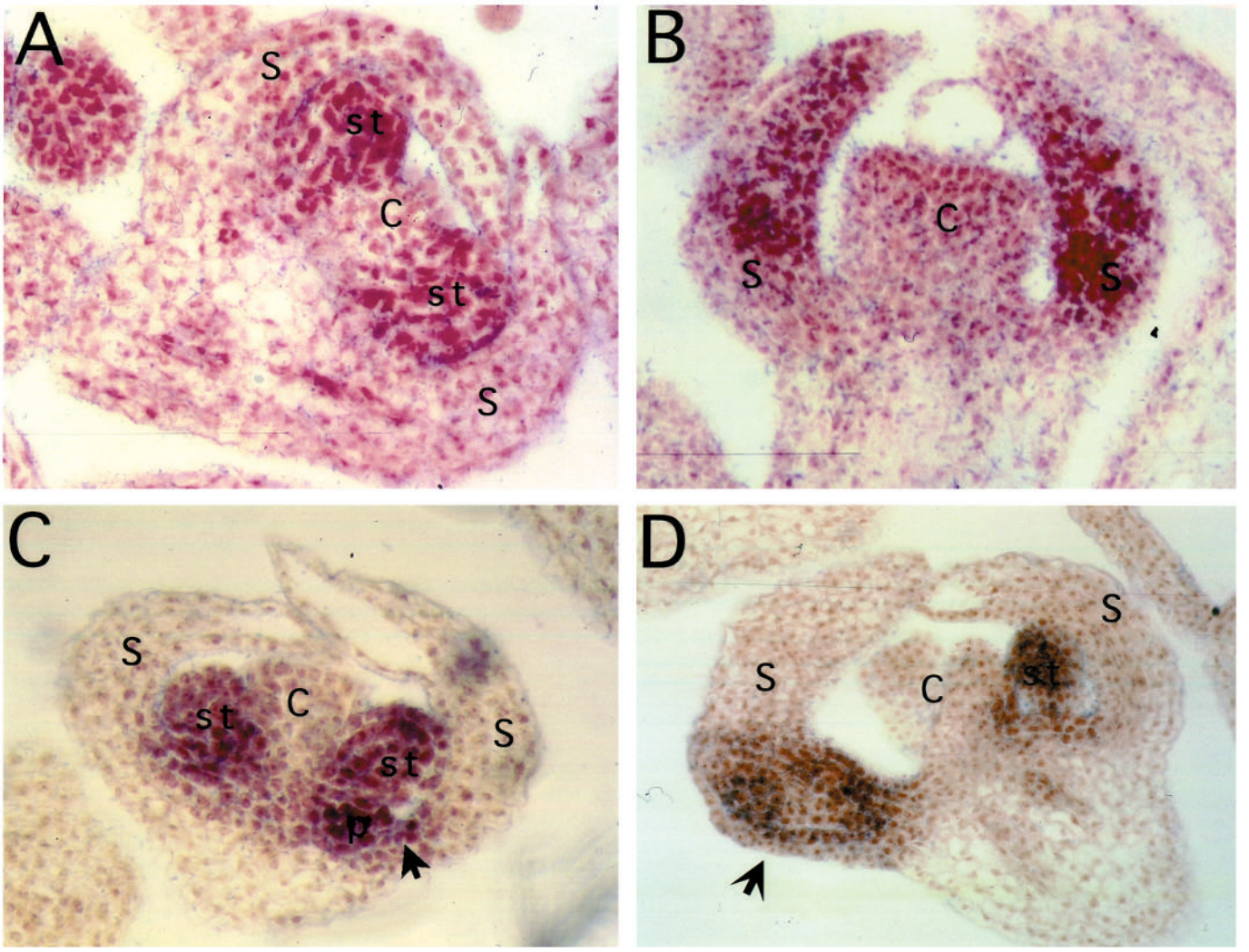


Fig. 4. Class B gene expression in wild-type and *lug-1* mutants. In situ hybridization of Dig (digoxigenin) labeled antisense probes of *PI* (A, B) or *AP3* (C, D) to 8 μm longitudinal sections of young wild-type and *lug* inflorescence apices (Materials and Methods). The images are bright-field microphotographs. (A) In this wild-type stage 6 flower, *PI* RNA is absent from sepal (S) and carpel (C) primordia but is detected in the stamen (st) and petal primordia (petal is small and absent from this section). (B) *PI* RNA is detected in both sepals of this stage 7 *lug-1* flower. The stamen and petal primordia are severely reduced in number, and thus missing from this section. (C) In this wild-type stage 7 flower, *AP3* RNA is absent in sepal (S) and carpel (C) primordia but is detected in the stamen (st) and petal (P) primordia. *AP3* RNA is also detected at the base of the sepal (arrow). (D) *AP3* RNA is detected in one of the sepals (arrow) in this stage 7 flower of *lug-1*.

subfunctions: specification of sepal and petal identity and repression of *AG* expression. The three class A genes *LUG*, *AP2*, and *AP1* are distinct from one another with respect to these two subfunctions (Fig. 9B). *AP1* is required for organ identity specification but not for *AG* repression (Mandel et al., 1992; Gustafson-Brown et al., 1994). *LUG* is required for *AG* repression but not for the organ identity specification (this study). *AP2* is required for both organ identity specification and *AG* repression (Bowman et al., 1991).

However, our study also suggests that *AP1* is likely a redundant repressor of *AG*. *ap1-1* enhances floral homeotic transformation as well as *AG* misexpression in *lug-1 ap1-1* double mutants, indicating a role for *AP1* in *AG* repression in the absence of *LUG*. Similarly, in the absence of *AP2*, *AP1* contributes to *AG* repression as indicated by the enhanced homeotic transformation in outer whorl organs of *ap1-1 ap2-1* double mutants (Bowman et al., 1993). This is consistent with the observation that *AG* is occasionally expressed in whorl 1 organs of *ap1* mutants (Weigel and Meyerowitz, 1993), and that carpelloid whorl 1 and staminoid whorl 2 organs are sometimes observed in *ap1* mutants (Bowman et al., 1993; Schultz and Haughn, 1993).

The weak *ap2* allele, *ap2-1*, is defective in sepal and petal specification (Bowman et al., 1991) but retains, at least partially, the subfunction for *AG* repression, because *AG* RNA distribution is still restricted to whorls 3 and 4 of the *ap2-1* flowers (Fig. 3A; Drews et al., 1991). The *lug-1* single mutation causes only 58% of whorl 1 organs to misexpress *AG* (this study). However, *ap2-1 lug-1* flowers exhibit enhanced floral homeotic transformation as well as 100% *AG* misex-

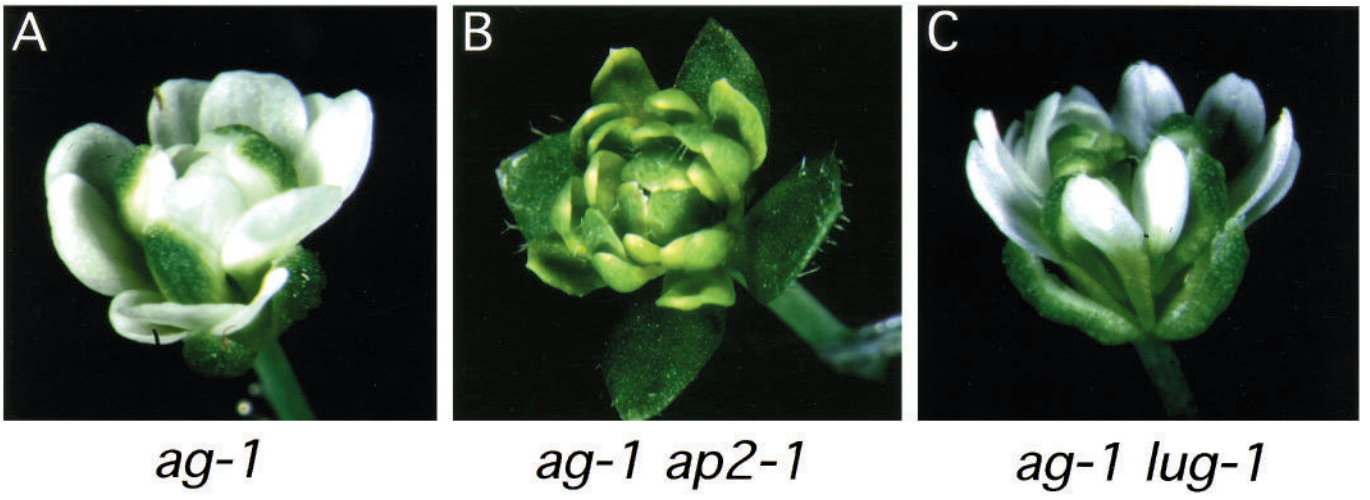


Fig. 5. The *ag-1 lug-1* flower is similar to *ag-1* but is distinct from *ag-1 ap2-1* flowers. (A) An *ag-1* flower. Whorls 1 and 2 are similar to wild-type consisting of 4 sepals and 4 petals respectively. Whorl 3 consists of six petals, and whorl 4 consists of a new flower. (B) An *ag-1 ap2-1* flower. Whorl 1 consists of 4 leaves; whorls 2 and 3 give rise to 4 and 6 organs intermediate between petals and stamens (Bowman et al., 1991); whorl 4 is another flower of the same phenotype. (C) An *ag-1 lug-1* flower. Similarly to *ag-1* (see A), whorl 1 and 2 are 4 sepals and 4 petals respectively. Whorl 3 consists of 6 petals, and whorl 4 is another flower. The sepals and petals are narrow in shape.

pression in whorl 1, indicating that *ap2-1* and *lug-1* each enhances the other's defect in *AG* repression. This synergy between *ap2* and *lug* is also made evident by the dominant interaction between *lug-1/lug-1* and *ap2-9/+*, and could be explained by two alternative mechanisms: (1) a threshold level of an activity composed of *LUG* and *AP2* may be required for *AG* repression, *lug-1/lug-1 ap2-9/+* plants possess a level of activity much lower that of *lug/lug +/+*, and this difference in the activity level is manifested in their differences in phenotypes; or (2) *AP2* and *LUG* proteins may form heteromultimeric complexes for *AG* repression. A copy of the mutant *ap2* protein may dramatically affect the activity of the complex as in the case of dominant negative types of interactions (Herskowitz, 1987).

Since the phenotype of double mutant *lug-1 ap2-9* is more severe than the phenotype of the strongest reported *ap2* allele, *ap2-2*, *LUG* and *AP2* are partially redundant in their A cadastral function. However, *LUG* appears to play a relatively minor role compared to *AP2* with respect to this A cadastral activity. This conclusion is based on two observations: that all *lug* mutations cause incomplete and less severe homeotic transformation than *ap2* mutants, and that *lug* mutations are recessive in an *ap2-9/ap2-9* background.

Other roles of *LUG*

Under our growth conditions, cauline leaves and late rosette leaves of *lug* are narrower and more serrated than wild type. *lug* floral organs are also narrower and more pointed than in wild-type. This effect of *lug* on leaf and floral organ shape is independent of the activities of *AG, AP2, AP1, PI, AP3, sup* and *LFY;* thus *LUG* may directly or indirectly regulate genes specifying organ shape.

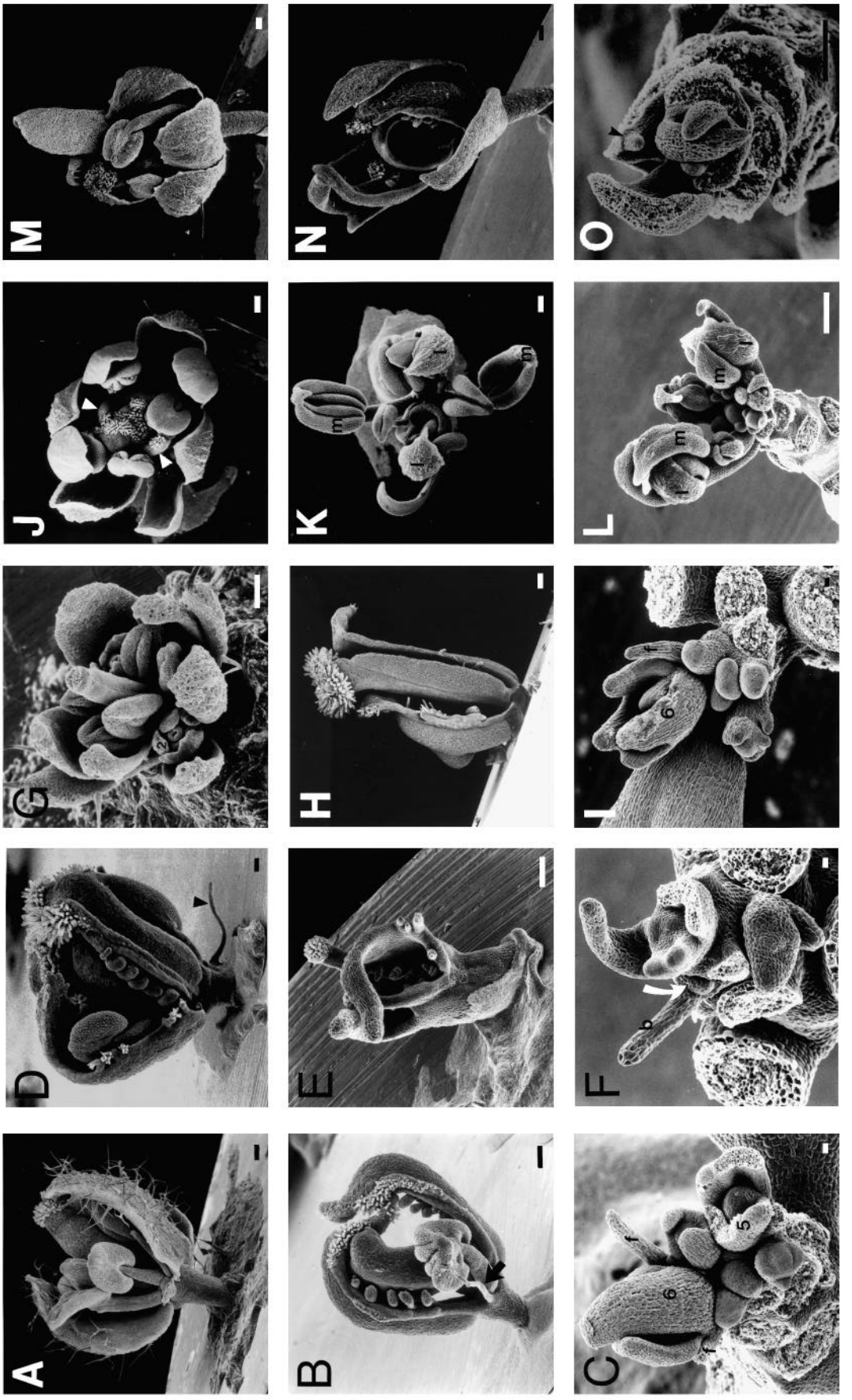
LUG is essential for proper carpel fusion and septum formation. The horn-like protrusion at the tips of carpels may prevent the fusion of carpels by continuous outgrowth. These horn-like protrusions are also found in *lug* mutants whorl 1 carpelloid organs in *lug*, and have never been observed in wild-type. This horn-like protrusion in *lug* is found in all double mutants examined, including those of *lug* with *ap2, ap1, pi, ap3*, and *lfy*. It is possible that this defect of *lug* is associated with the similar defects in the shape of leaf and floral organs.

The diverse defects of *lug* mutants suggest that *LUG* may be involved in regulating several different developmental processes, in which *LUG* may interact with different partners.

Is *LUG* the predicted cadastral gene?

Several lines of evidence suggested the existence of factors other than *AP2* for *AG* repression. First, *AP2* RNA is expressed in all four whorls of a flower (Jofuku et al., 1994), yet *AP2* only represses *AG* expression in whorls 1 and 2 (Drews et al., 1991). Thus the ability of *AP2* to repress *AG* must depend on additional spatially specific factor(s). Second, when *ag* mutations cause ectopic *AP2* organ identity function in whorls 3 and 4, they do not cause ectopic *AP2* cadastral activity because *AG* mutant RNA is correctly expressed in whorls 3 and 4 (Gustafson-Brown et al., 1994). This again suggests the existence of at least one additional factor, whose activity is spatially-restricted regardless of *AG* activity, and whose activity is neither required for, nor interferes with, sepal and petal specification, because normal sepals and petals are ectopically formed in whorls 3 and 4 of *ag* mutants. *LUG* could be a candidate for such a factor, for it is clearly required for *AG* repression in whorls 1 and 2 and it is not required for sepal and petal specification.

Nonetheless, the roles of *LUG* in regulating leaf and floral organ shape as well as its role in regulating stamen number and carpel fusion indicate that *LUG* is active, at least at some developmental stages, in vegetative parts of plants and in whorls 3 and 4. Thus, *LUG* may not be a spatially-restricted whorl 1 and 2 factor. It is likely that *LUG* is part of the class A cadastral complex just as *AP2* may be part of the class A cadastral complex. The molecular cloning of *LUG*, and the consequent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ability to detect and change its region and time of expression should help clarify this issue.

lug indirectly alters the domain of B gene activity

The ectopic B class activity in *lug* mutants appears to be an indirect effect through ectopic *AG* activity because eliminating *AG* in *ag-1 lug-1* double mutants greatly reduces ectopic B activity. One explanation is that both *AP1* and *AP2* activities are repressed by ectopic *AG* in whorl 1 organs of *lug* mutants, and either *AP1* or *AP2* activity is required to prevent *PI* or *AP3* from being expressed in whorl 1 (Fig. 9A). This is supported by the observation that *ap1-5*, a weak *ap1* mutation, exhibits petaloid sepals at the medial position in whorl 1 (Bowman et al., 1993). Similarly, *ap2-8* and *ap2-9* exhibit staminoid features in whorl 1 organs at the medial position (Bowman et al., 1991). An alternative explanation is that ectopic *AG* directly causes ectopic B activity in whorl 1 of *lug* mutants. For instance, *AG* may cause medial sepal primordia to arise closer to the region of whorl 2 in a floral primordium, and as a result, the medial whorl 1 organs are more likely to express B activity.

Common and unique properties of *lug, ap1* and *ap2* mutants

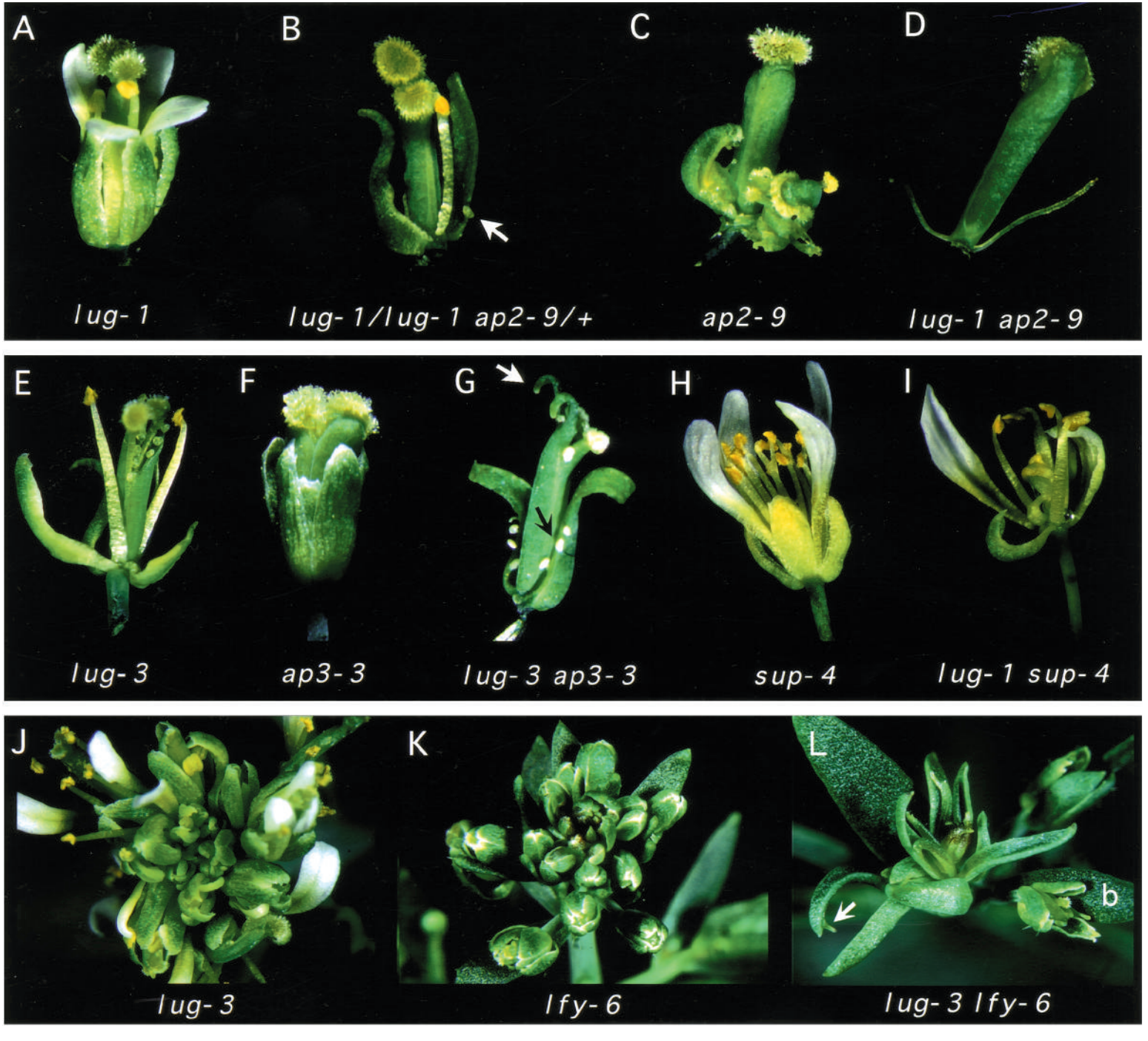
The combined functions of *LUG*, *AP1* and *AP2* contribute to the so-called A function. Mutations in any one of these three genes affect A function to certain degrees and thus exhibit similarities as follows: (1) flowers at more apical positions have more severe phenotypes; (2) the medial whorl 1 organs are more readily transformed into carpelloid and staminoid sepals than lateral whorl 1 organs, which are more likely to develop into sepals, leaves, filaments, or to be absent; (3) all class A mutants have a reduced number of whorl 2 and 3 organs due to the failure of organ initiation. These similarities might be attributed to changes in *AG* activity: (1) endogenous *AG* activity may increase apically; (2) medial whorl 1 organs might be more susceptible to ectopic *AG* or the lateral whorl 1 organs might reside outside the influence of organ identity genes because they initiate at a lower position in a floral meristem (Smyth et al., 1990; Bowman et al., 1991, 1993); (3) the loss of whorl 2 and 3 organs can be attributed, at least in part, to ectopic *AG* activity, because removing *AG* activity in any of the A class mutants can recover some or most of the lost organs (Irish and Sussex, 1990; Bowman et al., 1991, 1993; Weigel and Meyerowitz, 1993).

lug, *ap1* and *ap2* mutants also exhibit unique properties. For instance, *lug* affects leaf and floral organ shape and septum fusion, *ap1* causes axillary flowers in the axils of whorl 1 bracts, and enhances defects of *lfy* in floral meristem identity specification (Irish and Sussex, 1990; Weigel et al., 1992; Bowman et al., 1993), and *ap2* causes abnormal seed coats (Leon-Kloosterziel et al., 1994; Jofuku et al., 1994). These differences make it unlikely that one class A gene is strictly an upstream regulator of another class A gene. It is possible, however, that one gene regulates the expression of another gene at a specific time or in specific tissues as is the case where *AP1* RNA expression in a stage 2 and later floral primordium is regulated indirectly by *LUG* (this study).

Flower development in other plant species

Studies on flower development in snapdragon, petunia, tobacco, and tomato indicated that despite variations in floral form and size, the essential mechanisms underlying the development of floral ground plan are similar (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). Floral homeotic genes or floral homeotic mutants with A, B, and C functions are found in several plant species studied (Carpenter and Coen, 1990; Schwarz-Sommer et al., 1990; Sommer et al., 1992; Angenent et al., 1993; van der Krol et al., 1993; Pnueli et al., 1994). Similarly to *lug* and *ap2* in *Arabidopsis*, the *blind* (*bl*) mutation in *Petunia hybrida* causes ectopic expression of *pMADS3*, a homologue of *AG* and *PLE*, in all floral whorls and in leaves (Tsuchimoto et al., 1993). However, *bl* mutants only exhibit homeotic conversions of corolla limb to antheroid structures (de Vlaming et al., 1984; Angenent et al., 1993; Tsuchimoto et al., 1993). *ovulata* (*ovu*) in *Antirrhinum majus* (snapdragon) is a dominant gain-of-function mutation, found to be due to a transposon insertion within *PLENA* (*PLE*), the snapdragon class C gene. This insertion also causes ectopic expression of *PLE* and thus ectopic C function in whorls 1 and 2 (Bradley et al., 1993). Understanding how domain-specific activities of floral homeotic genes are regulated in *Arabidopsis* and other plant species will shed light on the evolution of one key control mechanism in floral pattern formation.

Fig. 6. Scanning electron microscopic (SEM) pictures of single and double mutants. Bars equal 10 μm in C, F, and I; bars equal 100 μm in the remaining photos. Numbers indicate the stages of respective flowers. Abbreviations are: f, filament; b, bract; m, medial; l, lateral; 2°, secondary flower. (A) An *ap2-1* flower at 29°C. One first whorl leaf is removed to reveal interior organs. (B) A *lug-1 ap2-1* flower with two medial whorl 1 carpels and two lateral whorl 1 filaments (arrow). Whorl 2 organs are completely absent, whorl 3 has one stamen, and whorl 4 has an abnormal gynoecium. (C) An inflorescence of a *lug-1 ap2-1* double mutant. A basal stage 6 flower (6) is flanked by two lateral filaments (f) (one of which was removed). These filaments are not observed in mature flowers (see B), and thus are aborted later. In the stage 5 flower (5), the fused whorl 1 organs were dissected away to reveal the flat central dome and the absence of whorl 2 organ primordia. (D) An *ap2-9* flower with a similar phenotype to *lug-1 ap2-1* (see B). (E) A *lug-1 ap2-9* flower consisting of one gynoecium. The gynoecium is unfused and has horn-like protrusions. (F) An inflorescence of *lug-1 ap2-9*. Each floral primordium consists of a bract (b) subtending a flat dome (arrow), which will develop into a single gynoecium. The bract is filament-like and frequently aborts. (G) An *ap1-1* with axillary flowers (2°) developing in the axils of whorl 1 sepals. (H) A *lug-1 ap1-1* flower. Similarly to *lug-1ap2-1* (see B), the two medial whorl 1 organs are carpelloid, and the two lateral whorl 1 organs are absent or filaments. Whorl 2 and 3 organs do not develop. Axillary flowers are absent. (I) An inflorescence of genotype *lug-1 ap1-1*. Similarly to *lug-1 ap2-1* (see C), flowers have flanking lateral filaments (f) that abort later. Whorl 1 organs sometimes fuse with one another as shown in the stage 6 flower. Whorls 2 and 3 organs do not develop. (J) A *35S-AP3/+* flower. Whorls 1, 2 and 3 organs are wild-type, and whorl 4 consists of stamens with stigmatic papillae on top (arrows). (K) A basal *lug-1/lug-1 35S-AP3/+* flower. The two medial whorl 1 organs (m) are stamens, while the two lateral whorl 1 organs (l) are sepals. The number of organs in whorls 2 and 3 is severely reduced. (L) An inflorescence of *lug-1/lug-1 35S-AP3/+* showing the staminoid medial whorl 1 organs (m). (M) A *lfy-5* flower. (N) A *lfy-5 lug-1* flower. All organs (except the two innermost carpels) consist of sepal/carpel mosaic tissues. The few floral organs arise in a partially spiral pattern. (O) A young *lfy-5 lug-1* inflorescence terminating with bracts and carpelloid bracts (arrow indicates a developing ovule).


A
lug-1
B
lug-1/lug-1 ap2-9/+
C
ap2-9
D
lug-1 ap2-9
E
lug-3
F
ap3-3
G
lug-3 ap3-3
H
sup-4
I
lug-1 sup-4
J
lug-3
K
lfy-6
L
b
lug-3 lfy-6

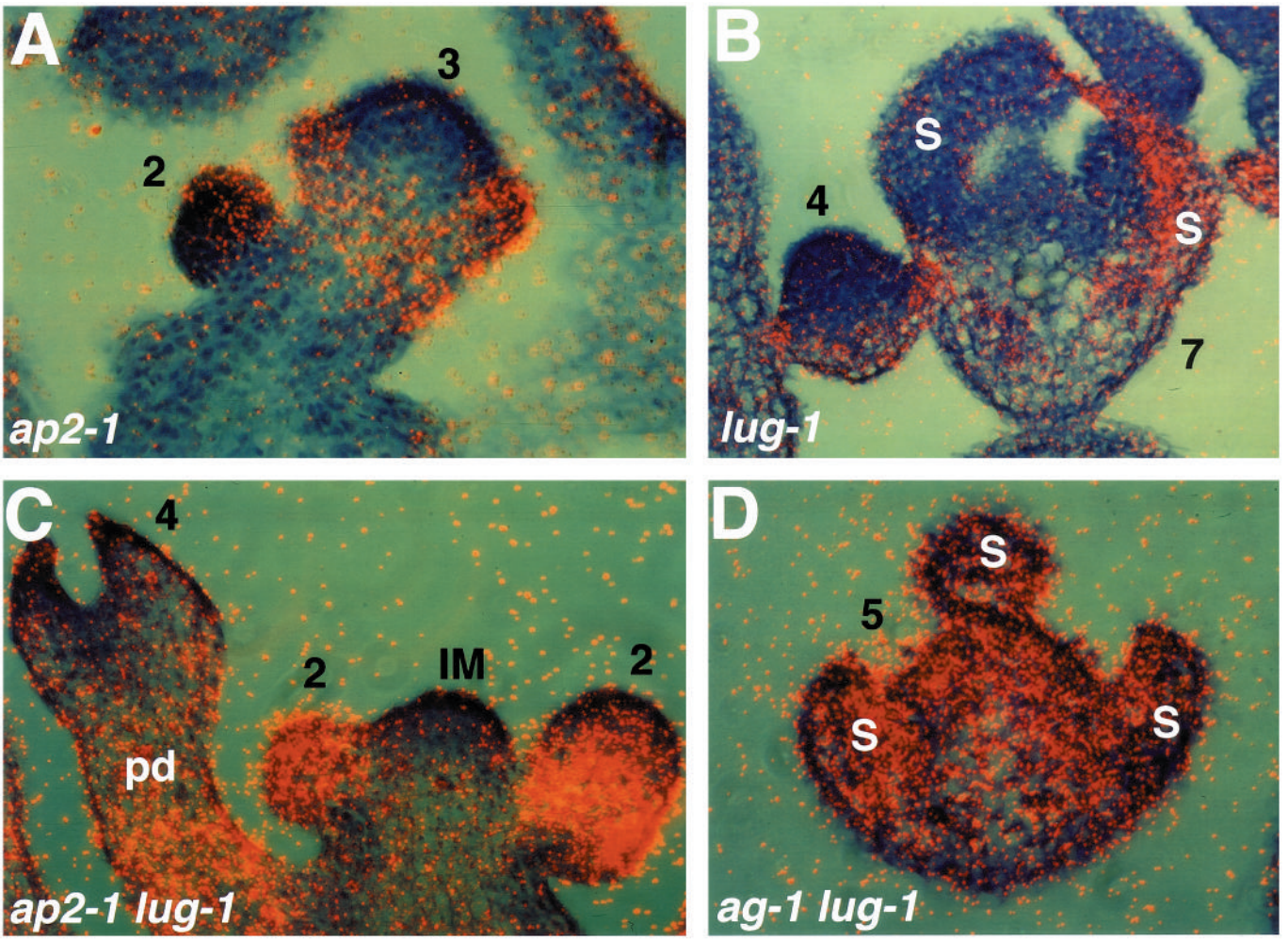
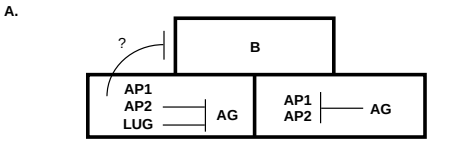


Fig. 8. *AP1* expression in single and double mutants. In situ hybridization of a radioactive (S^{35})*AP1* antisense probe to 8 μm longitudinal sections of plant inflorescence apices. The flowers shown are at apical positions 10-20th. The tissue staining and photography are described in Fig. 3 legend. The number indicates the stages of corresponding flowers according to Smyth et al. (1990). Abbreviations are: IM, inflorescence meristem; pd, pedicel. (A) *AP1* expression in *ap2-1*. Similarly to wild-type (Gustafson-Brown et al., 1994), *AP1* RNA is detected early in the entire floral primordium as shown in the stage 2 flower. At stage 3, *AP1* RNA is detected in the region where whorl 1 and 2 organs will develop. (B) *AP1* expression in a stage 4 (left) and a stage 7 (right) *lug-1* flower. *AP1* RNA is detected in only one of the two sepals (S) of the stage 7 flower. (C) *AP1* expression in flowers of genotype *ap2-1 lug-1*. Shown is an inflorescence meristem (IM) flanked by an early stage 2 (left), a late stage 2 (right), and a stage 4 flower. At the beginning, *AP1* expression is identical to that of wild-type as shown in the early stage 2 flower (left to the IM). In the late stage 2 (right) and the stage 4 flowers, *AP1* RNA is not present in floral meristems but remains in the pedicels (pd). (D) *AP1* expression in a stage 5 flower of genotype *ag-1 lug-1*. *AP1* RNA is detected in all sepals (S) (due to the angled section, three sepals are shown here). *AP1* RNA is also detected in the central dome that will give rise to whorls 2, 3 and 4.

Fig. 7. Double mutant combinations between *lug* and *ap2*, *ap3*, *sup*, and *lfy*. The top panel illustrates a increase in the severity of phenotype by losing one or more copies of wild-type *AP2* and *LUG*. (A) A basal *lug-1* flower (second flower) with little if any homeotic transformation. (B) A basal *lug-1/lug-1 ap2-9/+* flower (second flower). Medial whorl 1 sepals have ovules (arrow) developing along the margin. The whorl 2 organs are absent and whorl 3 stamens are reduced in number. (C) An *ap2-9* flower. The medial whorl 1 carpels exhibit both stigmatic papillae and ovules along the margins. Lateral whorl 1 organs are filaments or absent. Whorl 2 and 3 organs are not formed. (D) A *lug-1ap2-9* double homozygous flower. Almost all organs in whorls 1, 2 and 3 are absent. Occasionally, filaments are observed in whorl 1. The middle panel shows double mutants between *lug* and *ap3* or *sup*. (E) An apical *lug-3* flower. (F) An apical *ap3-3* flower. (G) An apical *lug-3 ap3-3* flower. In whorl 1, lateral organs are absent (as shown) or are filaments, medial organs are carpelloid with ovules developing along the margins (black arrow). Whorl 2 organs are absent, and the central gynoecium consists of roughly three carpels, one of which is probably a whorl 3 carpel. Note the horn-like (white arrow) protrusions associated with the carpels. (H) A *sup-4* flower. Whorls 3 and 4 consist of 12 stamens. (I) A *lug-1 sup-4* flower. Carpelloid sepals and slightly staminoid petals are made in whorls 1 and 2 respectively. Whorl 3 has reduced number of stamens; whorl 4 consists of stamens and staminoid carpels. Some sepals were removed to reveal the interior. The bottom panel illustrates inflorescences of *lug-3*, *lfy-6* and *lug-3 lfy-6* at comparable developmental stages. (J) A young *lug-3* inflorescence. At least 13 flowers are visible. (K) A young *lfy-6* inflorescence. At least 18 flowers are visible. (L) A young *lug-3 lfy-6* inflorescence. Only two flowers are visible. The inflorescence terminates early with bracts (b) or carpelloid bracts (arrow indicates carpelloid tissue). Note the narrow shape of leaves and bracts.



B.

Class A genes	Repress *AG*	Specify sepal and petal identity
AP1	-	+
LUG	+	-
AP2	+	+

Fig. 9. The role of *LUG* in floral organ identity determination. (A) The role of *LUG* in the ABC model. *LUG* is proposed to act together with *AP2* in whorls 1 and 2 for *AG* repression. *AP1* and *AP2* may negatively regulate B activity in whorl 1 (see Discussion on "*LUG* alters the domain of B gene activity"). (B) The class A genes can have two subfunctions: specifying sepal and petal identity and repressing *AG*. *AP1* only has the function for specifying sepal and petal identity, *LUG* only has a function for *AG* repression, and *AP2* possesses both functions for sepal and petals identity specification and for *AG* repression. "+" indicates a requirement for the respective gene and "–" indicates a non-essential role for the respective gene.

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