

# Regulation of Gynoecium Marginal Tissue Formation by *LEUNIG* and *AINTEGUMENTA*

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The carpel is the female reproductive organ of flowering plants. In *Arabidopsis*, congenital fusion of two carpels leads to the formation of an enclosed gynoecium. The margins of the two fused carpels are meristematic in nature and give rise to placentas, ovules, septa, abaxial repla, and the majority of the stylar and stigmatic tissues. Thus, understanding how the marginal tissues are specified and identifying genes that direct their development may provide important insight into higher plant reproductive development. In this study, we show that *LEUNIG* and *AINTEGUMENTA* are two critical regulators of marginal tissue development. Double mutants of *leunig aintegumenta* fail to develop placentas, ovules, septa, stigma, and style. This effect is specific to the *leunig aintegumenta* double mutant and is not found in other double mutant combinations such as *leunig apetala2* or *aintegumenta apetala2*. Additional analyses indicate that the absence of marginal tissues in *leunig aintegumenta* double mutants is not mediated by ectopic *AGAMOUS*. We propose that *LEUNIG* and *AINTEGUMENTA* act together to control the expression of common target genes that regulate cell proliferation associated with marginal tissue development.

## INTRODUCTION

Carpels, the female reproductive organ in flowering plants, are formed in the center of the flower, internally to stamens, petals, and sepals. The primary function of carpels is to generate and protect the developing ovules that, when fertilized, develop into seeds. Carpel identity has been shown to be specified by two separate pathways (reviewed in Bowman et al., 1999). One pathway is mediated by the C class floral homeotic gene *AGAMOUS* (*AG*), and the other pathway is mediated by the *SPATULA* (*SPT*) and *CRABS CLAW* (*CRC*) genes. Both pathways are negatively regulated by the A class genes such as *APETALA2* (*AP2*) and *LEUNIG* (*LUG*) (Bowman et al., 1991a; Drews et al., 1991; Liu and Meyerowitz, 1995; Alvarez and Smyth, 1999; Bowman and Smyth, 1999), thus restricting the development of carpel identity to organs in the center of a flower.

Subsequent to the determination of carpel identity, regional specification and differentiation of carpel tissues take place. Analyses of *ETTIN* (*ETT*) and *CRC* suggested the existence of apical–basal and abaxial–adaxial boundaries in carpel primordia (Sessions and Zambryski, 1995; Sessions, 1997; Sessions et al., 1997; Alvarez and Smyth, 1999; Bowman and Smyth, 1999; Eshed et al., 1999). In *ett* mutants, the alteration of carpel tissue distribution with respect to the apical–basal and abaxial–adaxial boundaries suggests that *ETT* plays a role in specifying or responding to regional domains.

In the case of *crc*, the isolation of its enhancers such as *GYMNOS* (*GYM*) establishes a role of *CRC* in specifying the abaxial region of the carpel that is not readily apparent from *crc* single mutants (Eshed et al., 1999).

In *Arabidopsis*, congenital fusion of two carpels leads to the formation of an enclosed gynoecium. The margins of the two fused carpels are medially situated with respect to the inflorescence. This medially situated and congenitally fused margin of the gynoecium exhibits a distinct developmental program. At early stages, when the gynoecial primordium is developing as a cylinder, the medially situated marginal tissue is active in cell division and gives rise to a ridge in the adaxial side of the gynoecium. This so-called medial ridge is meristematic in nature and apparently gives rise to placentas, ovules, and septa (Bowman et al., 1999). Hence, the medial ridge is of utmost importance for the female fertility of the plant. In addition, the medially situated and congenitally fused margin generates abaxial repla and contributes, at least partially if not mostly, to the formation of stigma and style. Hence, for simplicity and clarity, we use the term “medial ridge–derived tissues” to refer to the placentas, ovules, and septa and the term “marginal tissues” to refer to all of the medial ridge–derived tissues plus abaxial repla, stigma, and style. However, the laterally situated tissue of the gynoecium gives rise to the carpel walls (i.e., carpel valves) that protect the ovules within. Although lineage analyses are not yet available, the subdivision of a gynoecium into medial and lateral domains is supported by the expression of several genes that are specifically expressed either in the medial or in the lateral domain. For example, *FRUITFULL* (*FUL*)

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RNA is detected in the lateral domain and subsequently in carpel walls, whereas other *AGAMOUS-LIKE (AGL)* genes such as *AGL1* and *AGL5* are detected in the medial domain and subsequently in ovules, abaxial repla, and septa (Ma et al., 1991; Savidge et al., 1995; Flanagan et al., 1996; Gu et al., 1998; Bowman et al., 1999). Thus, the medial and lateral domains are distinguished by the patterns of both gene expression and function.

Although no mutation in *Arabidopsis* has been identified that specifically abolishes the medial or the lateral domain, numerous mutations have been isolated that cause various abnormalities in gynoecia, such as a reduced fusion between the two carpels and reduced septa, stigmatic, or transmitting tissues (reviewed in Bowman et al., 1999). Of particular interest are *aintegumenta (ant)* and *lug* mutants, both of which exhibit defects in carpel fusion (Komaki et al., 1988; Liu and Meyerowitz, 1995; Elliott et al., 1996; Klucher et al., 1996). In *ant* mutants, in addition to defects in carpel fusion, floral organ number in the first three whorls is reduced, and sepals, petals, and leaves are narrower than those in the wild-type plants (Elliott et al., 1996; Klucher et al., 1996). These defects can be attributed to smaller floral meristems (Krizek, 1999) and fewer cells in each of the lateral organs (Mizukami and Fischer, 2000). *ant* mutants are also female-sterile because of their severe defect in ovule integument initiation (Elliott et al., 1996; Klucher et al., 1996). *ANT* mRNA was detected in primordia of cotyledons, floral organs, placentas, ovules, and integuments of the ovules (Elliott et al., 1996; Long and Barton, 1998). Thus, both the mutant phenotype and the expression pattern suggest a role of *ANT* in promoting cell proliferation during the development of organ primordium.

Like *ant* mutants, *lug* mutants exhibit unfused carpels, narrow leaves and floral organs; fewer floral organs; and reduced female fertility (Liu and Meyerowitz, 1995). *lug* flowers exhibit homeotic transformations in which sepals are transformed into carpels or stamens and petals are transformed into stamens. The defect of *lug* in floral organ identity and floral organ number was previously shown to be caused by ectopic expression of *AG* mRNA. The reduced female fertility, on the other hand, was shown to be caused by abnormally overproliferating inner integument and a lack of embryo sac (Roe et al., 1997; Schneitz et al., 1997; Z. Liu and V.P. Klink, unpublished results).

Although homeotic transformation is rare in *ant* mutant flowers, the similarity between *lug* and *ant* mutants in other aspects of their phenotypes indicates that *LUG* and *ANT* may play similar roles during *Arabidopsis* development. In this study, we found that *lug ant* double mutants exhibit a strong synergistic interaction in the development of gynoecial marginal tissue. This interaction is specific to *lug ant* double mutants and is not found in other double mutant combinations between genes that encode repressors of *AG* expression. Our analyses provide important insight into *LUG* and *ANT* function as well as into the mechanism of gynoecium marginal tissue development.

## RESULTS

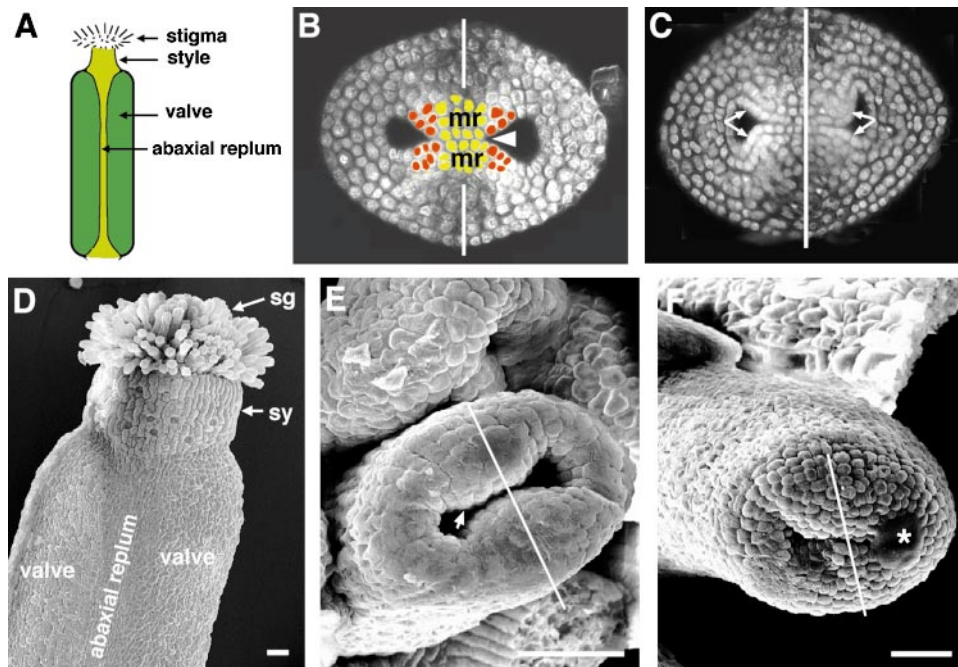
### Wild-Type Gynoecium Development

Figure 1 shows wild-type *Arabidopsis* gynoecium development. A gynoecium consists of two fused carpels and is capped by a single style and stigma (Figures 1A and 1D; Hill and Lord, 1989; Okada et al., 1989; Smyth et al., 1990; Gasser and Robinson-Beers, 1993; Sessions and Zambryski, 1995; Bowman et al., 1999). The two fused carpels are located laterally with respect to the inflorescence axis. These two carpels are congenitally fused, so the gynoecium initially develops as a slotted cylinder (Figures 1B, 1C, and 1E). At stage 9, as the cylinder starts to close at the apex, the apical epidermal cells differentiate into stigmatic papillary cells. These stigmatic cells first appear at the medial surface (Figure 1F) and then differentiate over the entire apical surface of the gynoecium. At stage 11, the style starts to differentiate just beneath the stigma (Figure 1D).

Inside the gynoecium cylinder, the medially situated marginal tissues give rise to two ridges that grow toward each other (Figures 1B, 1C, and 1E). These two medial ridges (Bowman et al., 1999) meet and then fuse together, resulting in a single septum (Figure 1B) that separates the gynoecium cylinder into a bilocular chamber. In addition, these two medial ridges give rise to four placentas and subsequently four rows of ovules (Figures 1B and 1C).

### Abnormal Gynoecium Development in *lug* and *ant* Single Mutants

Figure 2 illustrates gynoecium development in *lug* and *ant* single mutants. In *lug* single mutant gynoecia, abnormality is observed in both the lateral and the medial domains. First, the carpel valves grow excessively tall, forming hornlike protrusions at the apex (Figures 2A and 2B). These horns are not topped with stigmatic papillae, however; they are topped with cells that resemble cells of the style (cf. Figures 2F and 2G). Second, the postgenital fusion between the two medial ridges does not occur (Figures 2B and 2I); despite this failure in ridge fusion, however, ovules are formed from the placentas (Figures 2B and 2I). Hence, proper medial ridge fusion is not required for ovule formation. Third, the lateral and the medial domains are often unfused—either partially unfused near the apex (Figure 2C) or almost completely unfused (Figure 2A)—resulting in two horn-bearing carpel valves and two stigma/style/ovule-bearing medial structures (Figure 2A). Lack of valve fusion also does not affect the ability of the medial ridge to develop ovules (Figures 2B and 2J). The lack of congenital fusion can occur early (Figure 2D), when the two carpel valve primordia initiate and outgrow the two medial structures, or it can occur later in development (Figure 2C). The absence of stigma and a reduction in stylar tissue on top of the carpel valves whenever



**Figure 1.** Gynoecium Organization in Wild-Type Arabidopsis (*Landsberg erecta*).

**(A)** A diagram of a mature gynoecium. Two laterally situated carpel valves (dark green) are fused together. Abaxial replum is the joining area between the two carpel valves. The gynoecium is topped with a single style and stigma.

**(B)** An optical cross-section of a wild-type gynoecium (early stage 7). The staging throughout this study is based on Smyth et al. (1990). The tissue was stained with a DNA-staining dye, propidium iodide. The white line indicates the medial plane. The two inward outgrowths are the medial ridges (mr), which are initiated at the medial position. The medial ridges grow and fuse together to form a single septum (arrowhead). Cells of medial ridges are artificially colored with red and light green. The four red areas indicate the four placentas. Because lineage analyses are not yet available, the medial ridges are marked with approximations.

**(C)** An optical cross-section of a wild-type gynoecium at stages 7 to 8. Ovule primordia are evident in each of the four placentae indicated by the four arrows. The stronger propidium iodide stain in ovule primordia reflects greater cell division activity.

**(D)** A scanning electron microscopic (SEM) image of a mature wild-type gynoecium. The stigma (sg), style (sy), the two carpel valves, and the abaxial replum are indicated.

**(E)** An SEM image of a wild-type stage 7 gynoecium. The line indicates the medial plane, where medial ridges emerge (arrow).

**(F)** An SEM image of a wild-type stage 10 gynoecium. The line indicates the medial plane. The stigmatic papillar cells are starting to differentiate prominently at the medial apical surface. The asterisk indicates a dust contaminant during sample preparation.

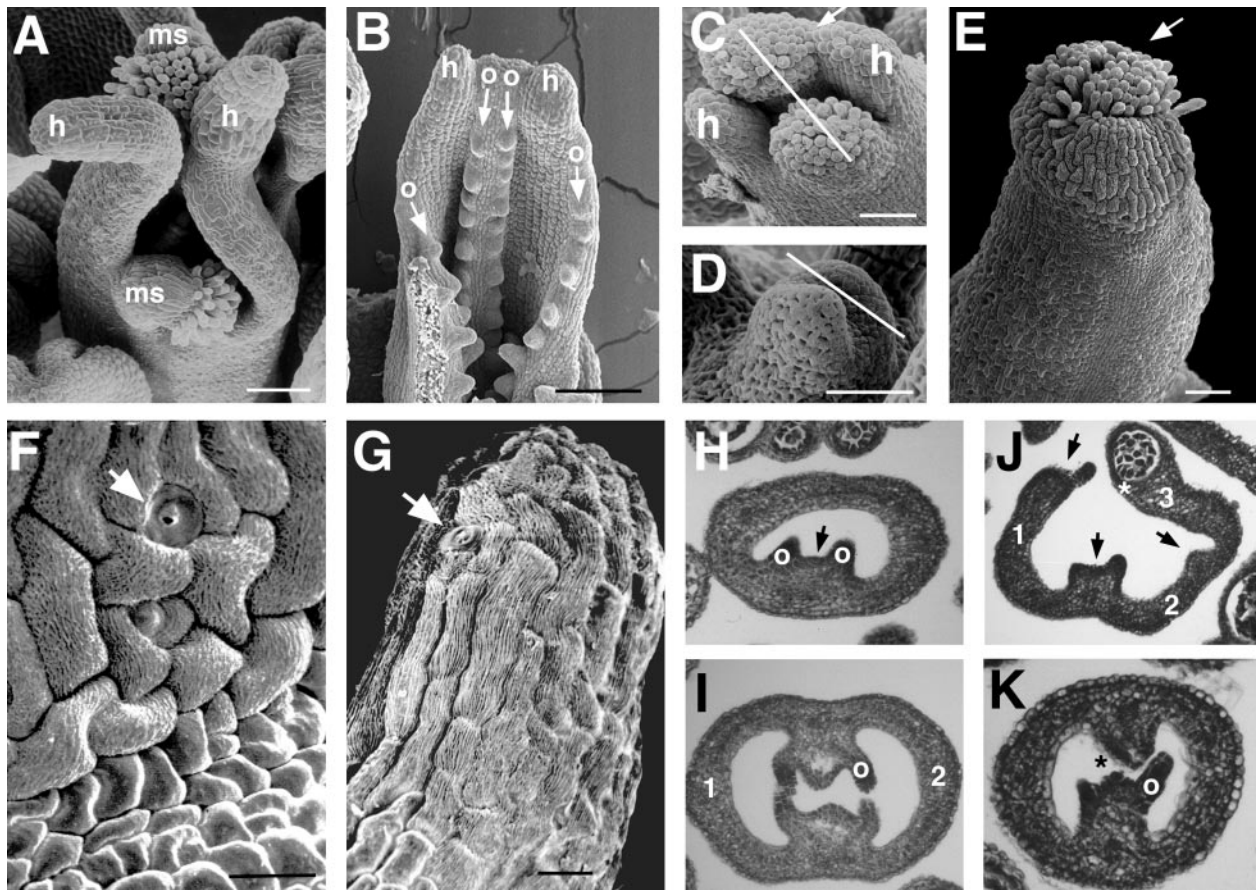
Bars in **(D)** to **(F)** = 20  $\mu$ m.

the carpel valves fail to fuse with medial structures (Figure 2A) suggest that the medial domain may play an important role in directing stigma/style formation. In addition to the abnormality in lateral and medial tissue development, the number of carpels in *lug* single mutants ranges from one to three (Figures 2H to 2J). In *lug-1* mutants, 25% of the gynoecia have one or 1.5 carpels, 71% of the gynoecia have two carpels, and 3.7% of the gynoecia have three carpels (Table 1). In *lug-3*, 75% of the gynoecia have one to 1.5 carpels, whereas only 25% of the gynoecia have two carpels (Table 1).

*ant-9* single mutant gynoecia also develop abnormally, albeit to a lesser extent. The stigmatic papillae are fewer and shorter than in the wild type (Figure 2E; Elliott et al., 1996).

*ant-9* medial ridges are frequently unfused to each other (Figure 2K; Klucher et al., 1996), and *ant* mutant carpels sometimes do not fuse near the apex (Elliott et al., 1996). Small horns are sometimes observed in *ant* single mutants (data not shown). Like *lug* single mutants, *ant-9* mutant gynoecia also consist of one to 1.5, two, and three carpels at 12.5%, 83%, and 4.1%, respectively (Table 1).

The number of ovules initiated in *lug-1*, *lug-3*, and *ant-9* single mutants is markedly decreased, as shown in Table 2. The average number of ovules per carpel in the wild type is  $26.4 \pm 1.3$ , whereas *ant-9* single mutants average  $14.8 \pm 4.4$  ovules per carpel. *lug-1* and *lug-3* mutants also show fewer ovules, averaging  $15.4 \pm 4.2$  and  $14.9 \pm 3.1$ , respectively (Table 2). Although 100% of *lug-1*, *lug-3*, and *ant-9*



**Figure 2.** Gynoecium Development in *lug* and *ant* Single Mutants.

(A) A *lug-3* gynoecium at stage 11 or 12. The two carpel valves and the two medial structures (ms) are not fused with each other. The valves are topped with horns (h), whereas the medial structures are topped with style and stigmatic papillae.

(B) A *lug-1* gynoecium unfused near the apex, revealing the developing ovules (o and arrows), despite failure in fusion. h, horn.

(C) A *lug-1* gynoecium. The line indicates the medial plane. Stigmatic papillae are prominently developing from the two medial swellings. One valve at the right is still fused to the medial swelling (arrow) and is developing some stigmatic papillae. h, horn.

(D) A stage 8 *lug-2* mutant gynoecium. This gynoecium is developing more slowly than the rest of the flower. The line indicates the medial plane. The two laterally situated protrusions (possibly protruding horns) represent the two carpel valves. Congenital fusion may be failing at this early stage.

(E) A stage 12 *ant-9* gynoecium, showing fewer and shorter stigmatic papillae (arrow) than the wild type (Figure 1D).

(F) A close-up image of the wild-type style, which consists of stomata cells (arrow) and cells exhibiting fine surface grooves.

(G) A close-up of the tip of a *lug-1* horn. The horn is topped with cells that harbor fine surface grooves and with stomata (arrow). These cells resemble the cells of the style in (F).

(H) A cross-section of a one-carpel *lug-1* gynoecium. There is only one medial ridge (arrow) and one carpel valve. Note the reduced septal tissue and the two ovule primordia (o).

(I) A cross-section of a *lug-1* gynoecium at stage 10. Postgenital fusion of the two medial ridges failed to occur. The two carpel valves are labeled 1 and 2. One of the four ovules (o) is indicated.

(J) A cross-section of a three-carpel *lug-1* gynoecium. 1, 2, and 3 indicate the three carpel valves. The three arrows indicate the three medial ridges. Carpel 3 is partially transformed into a stamen, with an anther locule labeled with an asterisk.

(K) A cross-section of an *ant-9* gynoecium at stage 10. The medial ridges fail to fuse with each other (\*). One ovule (o) is indicated.

Bars in (A), (B), and (E) = 50  $\mu$ m; bars in (C), (D), (F), and (G) = 20  $\mu$ m.

plants form stigma, style, and septa (Table 2), these marginal tissues are not necessarily normal, as described above. Hence both *lug* and *ant* single mutants exhibit various types and degrees of defects in gynoecium development.

### Gynoecium Development and Ovule Initiation in *lug ant* Double Mutants

We constructed double mutants of *lug* and *ant* to test for any redundant functions not revealed in either single mutant. Both *lug-1 ant-9* and *lug-3 ant-9* double mutants were constructed. *lug-1* is an intermediate-strength allele, whereas *lug-3* is a strong allele (Liu and Meyerowitz, 1995). The *lug-1* mutation is caused by a single base pair substitution that alters a splicing acceptor site (Conner and Liu, 2000). *lug-3* is caused by a nonsense mutation that results in early termination of the protein and is likely a null (Conner and Liu, 2000). *ant-9* is a strong allele caused by a transposon *Activator* (*Ac*) insertion into the second intron (Elliott et al., 1996). Figures 3 and 4 illustrate the defects observed in *lug-1 ant-9* and *lug-3 ant-9* double mutants. We found that in *lug-3 ant-9* double mutants, the fourth-whorl gynoecium consisted of just two horn-bearing carpel valves (Figures 3A and 4E). Each carpel valve had a lateral vascular bundle (data not shown), and none of the *lug-3 ant-9* carpels examined ( $n = 67$ ) formed any ovule (cf. Figures 3E and 3F; Table 2). Occasionally (five times in 26), nub-shaped protrusions were found at the edge of the *lug-3 ant-9* carpel valves (Figure 3C). The epidermal morphology of these nubs resembled that of horns rather than ovules (Figure 3C inset). Hence, neither the fourth-whorl nor the first-whorl carpels in *lug-3 ant-9* double mutants formed any ovule, septum, or stigma (Figures 3A to 3C, 3F, and 4E, and Table 2). Except for stylelike cells at the tip of the horns and on nubs, no style was present (Figures 3A to 3C and Table 2).

*lug-1* single mutants exhibit a phenotype less severe than *lug-3*, and *lug-1 ant-9* double mutant gynoecia also exhibit a less severe phenotype. In the gynoecia of *lug-1 ant-9* double mutants, marginal tissues were in most cases absent (Figure 3I). However, 47% of *lug-1 ant-9* gynoecia ( $n = 17$ ) developed smaller than normal medial ridges, and these smaller medial ridges gave rise to partially formed septal tissues (including transmitting tract) and a medial vascular

bundle (Figure 3J). Nevertheless, none of the *lug-1 ant-9* first and fourth-whorl carpels could develop ovules, or stigma, or style (except for the stylarlike cells at the tip of horns) (Table 2). Thus, the ovule-forming ability of the placenta and the development of style and stigma are more sensitive than septa to the simultaneous loss of *LUG* and *ANT*.

Scanning electron microscopy analyses of the epidermal morphology of the sterile fourth-whorl organs indicate that they are carpel valves (Figures 3D, 3G, and 3H). Therefore, the lack of ovules and stigma/style in the *lug ant* double mutants probably does not result from homeotic transformation of carpels into leaves or other sterile organs. Specifically, the abaxial epidermis in the mutant carpel valve consists of a patchwork of clusters of four to five cells that surround an immature unopened stomata and is thus indistinguishable from the abaxial epidermis of a wild-type valve (cf. Figures 3G and 3H). Additionally, rectangular cells, characteristic of the edge of carpel valves, develop on the edge of the fourth-whorl organs in *lug-3 ant-9* double mutants (Figure 3D). The cellular morphology of the adaxial surface of *lug ant* double mutant carpel valves and wild-type carpel valves is also similar (data not shown). The hornlike structures at the top of these sterile organs (Figure 3A) are consistent with their carpel valve identity, given that horns are found only in *lug* carpels or carpelloid sepals. Furthermore, the partially formed septal tissue and transmitting tract in the *lug-1 ant-9* double mutant gynoecia (Figure 3J) support their carpel identity.

In addition to defects in the gynoecium, *lug ant* double mutants exhibit enhanced defects in whorls 1 to 3 of the flowers. In *lug-3* single mutant flowers (Figures 4B and 4F; Liu and Meyerowitz, 1995), the sepals at medial positions are staminoid/carpelloid, whereas the sepals at lateral positions are relatively normal. Second-whorl petals can be absent or staminoid. Third-whorl stamens are fewer in number, with only two or three stamens usually found. *ant-9* single mutants also display fewer floral organs in whorls 1 to 3 (Elliott et al., 1996; Klucher et al., 1996). Under our growth conditions and in the Landsberg *erecta* (*Ler*) background, *ant-9* flowers typically develop four narrow sepals, four narrow petals, and four stamens, and homeotic transformations are rare (Figures 4D and 4G; Elliott et al., 1996). We found that *lug-3 ant-9* double mutants have narrower floral organs

**Table 1.** Number of Carpels per Gynoecium in the Wild Type and Mutants (%)<sup>a</sup>

Carpels/Gynoecium	Wild Type	<i>lug-1</i>	<i>lug-3</i>	<i>ant-9</i>	<i>lug-1 ant-9</i>	<i>lug-3 ant-9</i>
1–1.5	0	25	75	12.5	0	19.4
2	100	71	25	83	89	80.5
3	0	3.7	0	4.1	10.5	0
	(29)	(28)	(12)	(24)	(19)	(36)

<sup>a</sup>Numbers within parentheses indicate the number of gynoecia examined.

**Table 2.** Formation of Marginal Tissues in the Wild Type and Mutants

Characteristic	Wild Type	<i>lug-1</i>	<i>lug-3</i>	<i>ant-9</i>	<i>lug-1 ant-9</i>	<i>lug-3 ant-9</i>
Ovules/carpel <sup>a</sup>	26.4 ± 1.3 (13)	15.4 ± 4.2 (11)	14.9 ± 3.1 (11)	14.8 ± 4.4 (13)	0.0 ± 0 (40)	0.0 ± 0 (67)
Gynoecia that form stigma <sup>b,c</sup>	100% (22)	100% (11)	100% (29)	100% (17)	0% (8)	0% (16)
Gynoecia that form style <sup>b,c</sup>	100% (13)	100% (11)	100% (14)	100% (9)	0% (8)	0% (8)
Gynoecia that form septa <sup>b,d</sup>	100% (29)	100% (20)	100% (18)	100% (16)	47% (17)	0% (28)

<sup>a</sup>Nomarski microscopy of xylene-cleared gynoecia was used to count the number of ovules per carpel. Numbers in parentheses indicate the number of carpels examined.

<sup>b</sup>Numbers within parentheses indicate the number of gynoecia examined.

<sup>c</sup>Scanning electron microscopy analyses were used to visualize stigma and style.

<sup>d</sup>Light microscopy of tissue cross-sections was used to visualize septum formation.

than either of the single mutants (Figures 4E and 4H). As shown in Figures 3A and 4E, the number of first-whorl organs decreased, with most flowers developing three instead of four first-whorl organs. Moreover, these first-whorl organs are usually carpelloid with horns. The second-whorl organs are completely absent. The third whorl usually consists of one to four abnormal stamens. Occasionally, filaments are found in the first or the third whorl (Figures 3A, 3B, and 4E). The fourth whorl, as described earlier, consists of one, 1.5, or two carpel valves topped with horns (Table 1). Thus, like *lug ap2* and *ant ap2* (Liu and Meyerowitz, 1995; Elliott et al., 1996), *lug ant* double mutants exhibit a synergistic effect on the number and identity of the floral organs.

#### AG RNA Expression in *lug-1*, *ant-9*, and *lug-1 ant-9* Mutants

AG is a key regulatory gene for flower development with at least two important functions: the specification of carpel and stamen identity, and the control of floral determinacy (Bowman et al., 1989, 1991a). AG encodes a transcription factor of the MADS box family (Yanofsky et al., 1990). In wild-type plants, AG RNA is absent in the inflorescence meristem as well as in stage 1, stage 2, and early stage 3 floral meristems. AG mRNA is first detected in the center of a midstage 3 floral meristem and later is restricted to stamens and carpel primordia (Figure 5A; Drews et al., 1991). This expression of AG is sufficient to specify stamen and carpel identity. In addition, AG is expressed in the medial ridge, ovule primordia, the chalazal region of ovules, and later in the endothelium (Figures 5B and 5C; Bowman et al., 1991b; Reiser et al., 1995).

Because the defects in transformation of floral organ identity and organ number in *lug* mutants were caused by ectopic and precocious expression of AG (Liu and Meyerowitz, 1995), we tested whether abnormal AG expression was also responsible for the flower and gynoecial defects seen in the *lug ant* double mutants. Specifically, in situ hybridization was used to test directly whether AG RNA was expressed precociously, ectopically, or both in young floral meristems

of *lug-1 ant-9* double mutants. Further, the expression of AG in gynoecia was examined to test if a higher level of AG expression in the medial ridge, or an earlier AG expression preceding medial ridge initiation, or an ectopic AG expression in the entire gynoecium (including lateral and medial domains) could be responsible for the defects of marginal tissue development in *lug ant* double mutants.

Precocious and ectopic AG mRNA expression was observed in floral meristems and floral organ primordia of *lug-1* single mutants (Figure 5D; Liu and Meyerowitz, 1995). AG mRNA was detected precociously in stage 2 floral meristems and ectopically in sepal primordia of *lug* mutants. However, in the medial ridge of *lug-1* mutants, the expression of AG was decreased, as was the size of the medial ridge (Figure 5E). Finally, AG mRNA expression was unaffected in the chalazal region of the ovule in *lug-1* mutants (Figure 5F). In *ant-9* mutants, AG mRNA was also expressed precociously in stage 2 floral meristems (Figure 5G). In developing sepals, ectopic AG expression was occasionally but infrequently observed (data not shown). In the medial ridge and the chalazal region of the ovule in *ant-9* mutants, the expression of AG mRNA was similar to that in the wild type (Figures 5H and 5I). In floral meristems and floral organ primordia of *lug-1 ant-9* double mutants, both precocious and ectopic AG mRNA expression was observed (Figure 5J). In the *lug-1 ant-9* double mutant gynoecia examined in our in situ hybridization experiments, AG mRNA was not detected in the anticipated medial ridge areas (Figures 5K and 5L). Thus, AG mRNA expression conveniently marks the medial ridge tissue in the wild type, and its absence in the *lug-1 ant-9* gynoecia correlates with the absence of the medial ridge.

In the wild-type gynoecium at stage 5 and later, expression of AG mRNA subsides, becomes completely absent from carpel valves, and is restricted to the medial ridge (Figure 5B). This subsiding AG expression was similarly observed in *lug-1* and *ant-9* single mutants as well as in *lug-1 ant-9* double mutants (Figures 5B, 5E, 5H, and 5K), indicating that the loss of medial ridge in *lug ant* double mutants was not caused by prolonged or increased AG expression in the gynoecium.

### Ovule Formation in Double Mutants of Negative Regulators of AG

Because our genetic analyses revealed a redundant role in marginal tissue development for *LUG* and *ANT* (both negative regulators of *AG* mRNA expression in floral meristems and floral organ primordia), we examined medial ridge-derived tissues in double mutants of additional genes that are negative regulators of *AG*. The known *AG* repressors include *LUG*, *ANT*, *AP2*, and *CURLY LEAF (CLF)*. *AP2* is a class A floral homeotic gene involved in both the negative regulation of *AG* and the specification of sepal and petal identity (Bowman et al., 1991a; Jofuku et al., 1994). *CLF* is required for the negative regulation of *AG* in vegetative tissues and for maintaining proper *AG* expression at late stages of flower development (Goodrich et al., 1997). We examined ovule and placenta formation in *lug-2 ap2-2*, *ant-9 ap2-2*, and *lug-8 clf-2* double mutants. As shown in Figure 6, unlike *lug ant-9* double mutants, the *lug-2 ap2-2*, *ap2-2 ant-9*, and *lug-8 clf-2* double mutants all formed placentas and initiated ovule development. Furthermore, stigmatic and stylar tissues were detected, although they were reduced (data not shown). Thus, the absence of ovules and other marginal tissues is unique to *lug ant* double mutants and is not a property of all double mutants of negative regulators of *AG*.

## DISCUSSION

### *LUG* and *ANT* Are Critical Regulators of Marginal Tissue Development

We found that the ability to form ovules, septa, stigma, and style is only weakly affected in *lug* or *ant* single mutants but is strongly affected in the *lug ant* double mutants. Strong *lug ant* double mutations completely abolish the formation of placentas, ovules, septa, and stigma. Although stylelike cells are found at the tip of horns, the *lug ant* double mutants also lack a style. Our finding indicates that *LUG* and *ANT* play a critical role in the development of gynoecium marginal tissues. The strong synergistic interaction between *lug* and *ant* in gynoecium marginal tissues is not observed in other double mutants, including *ap2-2 lug-2*, *ap2-2 ant-9*, and *clf-2 lug-8* (Figure 6). Hence, *LUG* and *ANT* are unique among this group of *AG* repressors, and this effect must reflect a specific requirement of these two genes in the development of these tissues.

### The Mechanism of *LUG* and *ANT* Action in Medial Ridge Development Differs from That in Floral Organ Identity Determination

Previous studies indicated that in developing floral meristems and floral organ primordia, ectopic and precocious

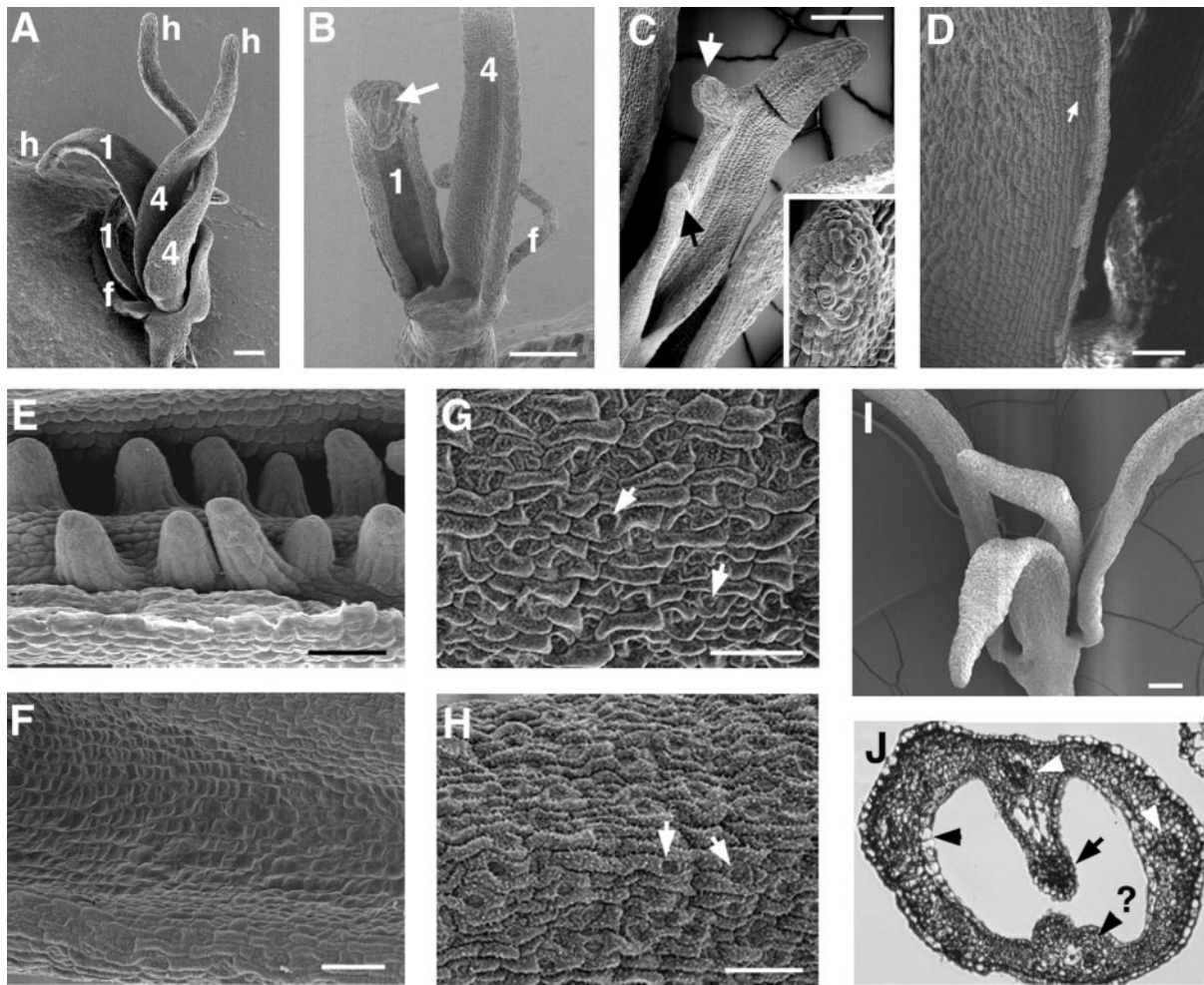
*AG* expression contributes to both homeotic transformation of floral organs and the reduction of floral organ number in *lug* mutants (Liu and Meyerowitz, 1995). In this study, we show that *AG* expression starts much earlier in *ant-9* mutant floral meristems than in the wild type. This precocious *AG* expression in *ant-9* may be at least partially responsible for the reduced floral organ numbers observed in *ant* mutant flowers. Thus, one function of *ANT* may be to prevent *AG* expression in very young floral meristems, to allow for sufficient cell proliferation and thereby regulate the number of cells in a floral meristem. The rare occurrence of ectopic *AG* expression in *ant-9* floral organ primordia explains the infrequent homeotic transformation observed in *ant* mutant flowers.

The defect in marginal tissue development, particularly in the medial ridge development, observed in *ant* single and *ant lug* double mutants, is consistent with a previously proposed role of *ANT* in regulating cell proliferation associated with organ primordial outgrowth. More recently, overexpression of *ANT* in *35S::ANT* transgenes was shown to cause enlarged embryos and bigger lateral organs in Arabidopsis and tobacco (Krizek, 1999; Mizukami and Fischer, 2000). Having observed that the increased cell number in the bigger organs of *35S::ANT* transgenic plants resulted from an extended period of cell proliferation, Mizukami and Fischer (2000) proposed that *ANT* regulates cell proliferation by maintaining the meristematic competence of cells during organogenesis. In addition, the importance of *ANT* in medial ridge formation is further supported by the recent work on *crc gym ant* triple mutants (Eshed et al., 1999) in which wild-type *ANT* activity is required for ectopic ovule formation on the abaxial surface of the *crc gym* mutant gynoecium. Because *ANT* encodes a protein with two DNA binding AP2 domains (Elliott et al., 1996; Klucher et al., 1996), it probably regulates the transcription of target genes with roles in cell proliferation associated with medial ridge development.

In contrast, the role of *LUG* in cell proliferation control is not well established. The narrower and smaller floral organs and leaves in *lug* single mutants are similar to *ant* single mutants and may thus reflect a role of *lug* in cell proliferation that is separate from its role in floral organ identity specification. The strong synergistic interaction between *lug* and *ant* in the medial ridge further supports a role of *LUG* in cell proliferation. We propose that *LUG*, a protein with WD repeats (Conner and Liu, 2000), may directly interact with the *ANT* protein to regulate the expression of common target genes in the medial ridge. Removing wild-type activities of both genes may considerably reduce the expression of these target genes and diminish cell proliferation activity in the medial ridge.

Several lines of evidence indicate that the absence of medial ridge formation and marginal tissues in *lug ant* double mutants is not mediated by ectopic *AG*. First, medial ridge formation still occurs in double mutants of *lug-2 ap2-2*, *ant-9 ap2-2*, and *clf-2 lug-8* (Figure 6), which exhibit similar homeotic transformation in floral organ identity and a decrease in organ number as a result of increased ectopic *AG* expression.





**Figure 3.** Gynoecium Marginal Tissues Are Absent in *lug ant* Double Mutants.

**(A)** The flower of a *lug-3 ant-9* double mutant having a total of five floral organs. The filament (f) represents a third-whorl organ. The other four organs all have characteristics of carpel valves, such as horns (h) and epidermal cell morphology. Two of these organs (both labeled 1) are first-whorl organs, and the other two organs (labeled 4) are fourth-whorl organs. Ovule, septum, stigma, and style are absent.

**(B)** A dissected *lug-3 ant-9* double mutant flower. Shown is the adaxial (inner) surface of a fourth-whorl (4) and a first-whorl (1) organ. A stamen filament (f) is visible. The first-whorl organ exhibits features of sepals as well as carpels; the long cell (arrow) on the abaxial surface is characteristic of sepals, whereas the thickening of the edge is characteristic of carpels. Ovule, stigma, and style were not detected in the first-whorl organs or in the fourth-whorl organs.

**(C)** A *lug-3 ant-9* double mutant fourth-whorl carpel. Nublike protrusions (arrows) at the edge of the valve display surface morphology characteristic of horns. The inset shows a magnification ( $\times 4$ ) of the nub (indicated by the black arrow).

**(D)** A close-up of the outer edge of an unfused *lug-3 ant-9* fourth-whorl carpel. The rectangular cells (arrow) are characteristic of cells at the edge of carpel valves.

**(E)** A close-up of the inner surface of a *lug-1* gynoecium. Two rows of ovules are initiated from the placenta.

**(F)** A close-up of the inner surface of a mature fourth-whorl organ in a *lug-3 ant-9* double mutant. Note the absence of any ovule primordium.

**(G)** A close-up of the abaxial epidermal surface of a wild-type *Ler* carpel valve. Immature stomata cells (arrows) and epicuticular wax are visible.

**(H)** A close-up of the abaxial epidermal surface of a *lug-3 ant-9* fourth-whorl organ. The immature stomata cells (arrows) and epicuticular wax (small white dots over the entire surface) are characteristic of carpel valves and are similar to the wild type, as shown in **(G)**. The more developed stomata and the more prominent wax than those in **(G)** reflect the older age of this flower.

**(I)** A *lug-1 ant-9* double mutant flower. None of the floral organs bears any marginal tissues.

**(J)** A cross-section of a stage 13 gynoecium of a *lug-1 ant-9* double mutant. No ovule is formed, although some septal tissue is present. Three vascular bundles are visible (arrowheads). The fourth (indicated by an arrowhead and a question mark) is less developed. A transmitting tract is also present (arrow).

Bars in **(A)**, **(B)**, **(C)**, and **(I)** = 200  $\mu\text{m}$ ; bars in **(D)** to **(H)** = 50  $\mu\text{m}$ .



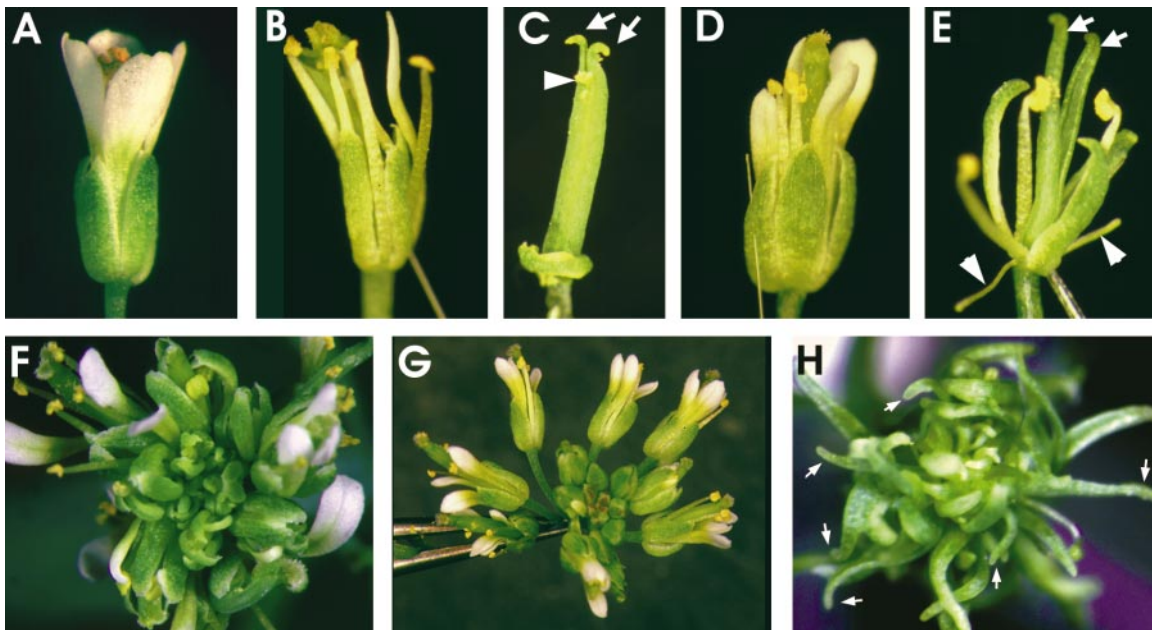
Second, transgenic plants that ectopically express *AG* under the control of a cauliflower mosaic virus 35S promoter can still initiate ovules in first-whorl as well as fourth-whorl carpels (Mandel et al., 1992; Mizukami and Ma, 1992; Ray et al., 1994), although these ovules are abnormal or carpel-like. Third, in situ examination of *AG* mRNA expression (Figure 5) revealed neither ectopic *AG* expression nor any abnormally prolonged *AG* expression in the gynoecial primordia, medial ridge, or the ovules of *lug-1* single, *ant-9* single, or *lug-1 ant-9* double mutants. Finally, because stigma and ovule still form in *ag ap2* double mutants (Bowman et al., 1991a; Alvarez and Smyth, 1999), *AG* activity is not essential for the development of these tissues.

#### Development of Medial and Lateral Domains Is Relatively Independent

Our study shows that eliminating the medial domain (as shown by a lack of any marginal tissues) does not drastically

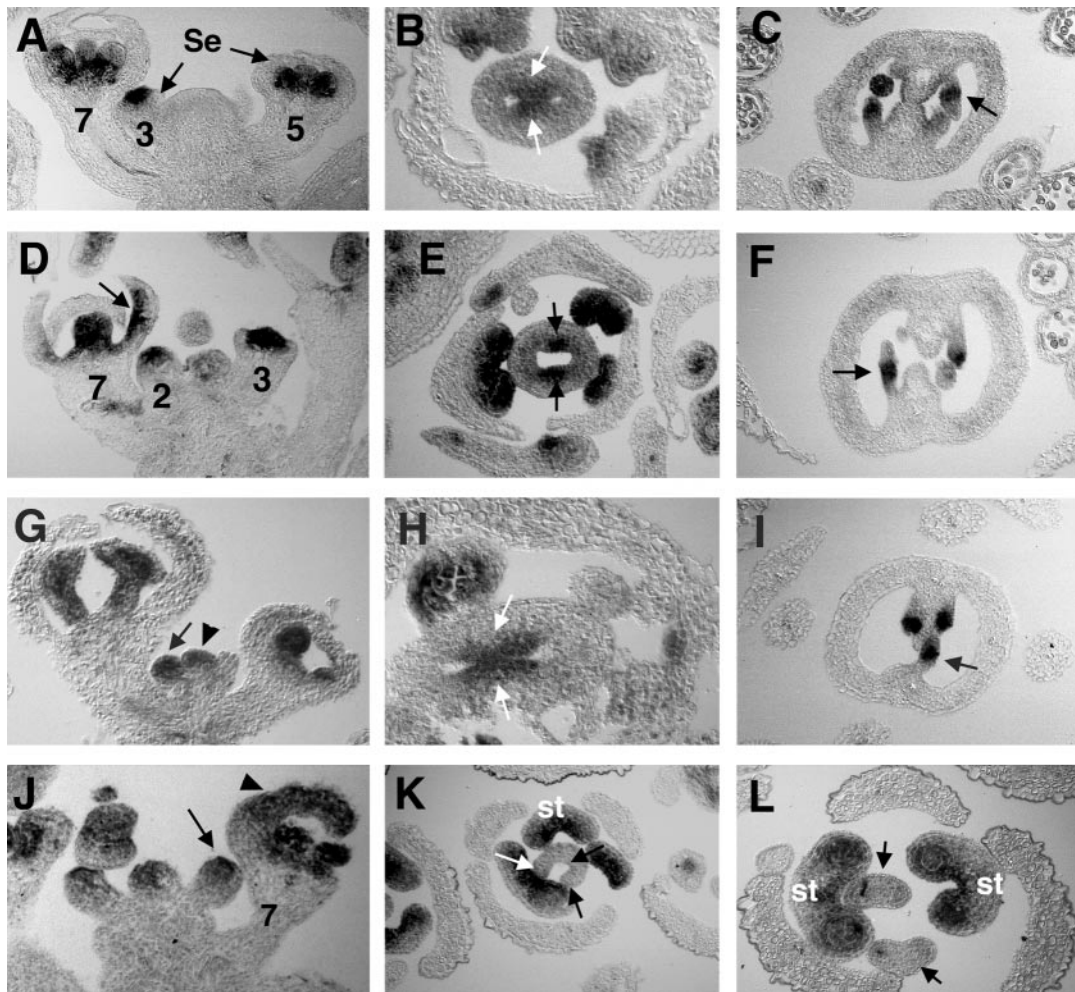
affect lateral domain development. Furthermore, the medially derived marginal tissues and the laterally derived carpel valves can develop and differentiate in the absence of fusion between them, suggesting that medial and lateral domains may develop relatively independently of each other.

In support of this notion, we note that *ett* mutants have a gynoecium defect almost complementary to that of *lug ant* double mutants. *ett-1* mutations cause an almost complete loss of valve tissue in the gynoecium (Sessions and Zambryski, 1995). Flowers of *ett tousled (tsl)* double mutants appear to have a stronger defect, in which the gynoecium consists of only a small mound of ovule-bearing tissue developing in the center of the flower lacking all carpel valve tissues (Roe et al., 1997). Perhaps the formation of these two types of gynoecium tissues—lateral carpel valve as opposed to marginal tissues—requires two different sets of genes: *ETT/TSL* and *LUG/ANT*, respectively. The former may promote carpel valve, whereas the latter may promote the marginal tissues. The interaction and fusion between these two tissues may further modulate gynoecium morphogenesis. Thus, in the



**Figure 4.** Floral Defects Are Enhanced in *lug ant* Double Mutants.

- (A) A wild-type flower.  
 (B) A *lug-3* flower. Floral organs are narrower than those in the wild type.  
 (C) A *lug-3* gynoecium with characteristic horns (arrows). The arrowhead indicates some stigmatic tissue.  
 (D) An *ant-9* flower.  
 (E) A *lug-3 ant-9* double mutant flower. There are three staminoid/carpelloid first-whorl sepals, two filaments (arrowheads), and three third-whorl stamens. No petal is formed. The two fourth-whorl organs do not fuse, are topped with horns (arrows), and are devoid of any marginal tissues.  
 (F) A *lug-3* inflorescence.  
 (G) An *ant-9* inflorescence.  
 (H) A *lug-3 ant-9* inflorescence. Note the many carpelloid first-whorl and fourth-whorl organs with horns (arrows). Floral organs are narrower than either *lug-3* or *ant-9* single mutants.



**Figure 5.** AG mRNA Expression in the Wild Type, *lug-1* and *ant-9* Single Mutants, and *lug-1 ant-9* Double Mutants.

**(A) to (C)** AG expression in the wild type (*Ler*). **(A)** AG is expressed in the center of a stage 3 flower and in the stamen and carpel primordia of flowers at later stages. Numbers indicate the stages of flowers, based on Smyth et al. (1990). Se, sepals. **(B)** AG is expressed in the medial ridge (arrows) but is absent in carpel valve in this stage 8 flower. **(C)** AG is expressed in the chalazal region (arrow) of the developing ovules. Integuments are initiated from this region.

**(D) to (F)** AG mRNA expression in *lug-1* mutants. **(D)** AG is ectopically expressed in the inner surface of a sepal in a stage 7 *lug-1* flower (arrow). AG is expressed precociously in a late stage 2 flower. **(E)** AG is expressed in the medial ridge in a stage 7 *lug-1* flower. Note the reduced AG expression as well as the reduced medial ridges (arrows). **(F)** AG is expressed in the chalazal area (arrow) of developing *lug-1* ovules.

**(G) to (I)** AG expression in *ant-9* mutants. **(G)** AG is precociously expressed in a midstage 2 *ant-9* floral meristem (arrow). The stage of this floral meristem was verified by examining adjacent sections. The meristem (arrowhead) at the center is a floral meristem rather than an inflorescence meristem. No ectopic expression of AG was found in this section. **(H)** AG is expressed in the medial ridge (arrows) of an *ant-9* gynoecium. **(I)** AG is expressed in the chalazal area (arrow) of developing ovules in *ant-9*.

**(J) to (L)** AG mRNA expression in *lug-1 ant-9* double mutants. **(J)** AG is precociously expressed in a stage 2 floral meristem (arrow) in *lug-1 ant-9* double mutants. Ectopic AG expression is detected in the developing sepal of a stage 7 flower (arrowhead). **(K)** AG expression is absent in the gynoecium of a stage 7 *lug-1 ant-9* double mutant flower. Arrows indicate the areas in which AG expression and medial ridge formation would have occurred in wild-type flowers. Nevertheless, AG expression is readily detected in the three developing stamens (st) of the same *lug-1 ant-9* flower. **(L)** Another *lug-1 ant-9* flower, showing two carpel valves (arrows) completely unfused to each other. No ovule is formed; neither are any medial ridge-derived tissues. The two carpel valves are surrounded by two stamens (st), which are surrounded by three sepals.

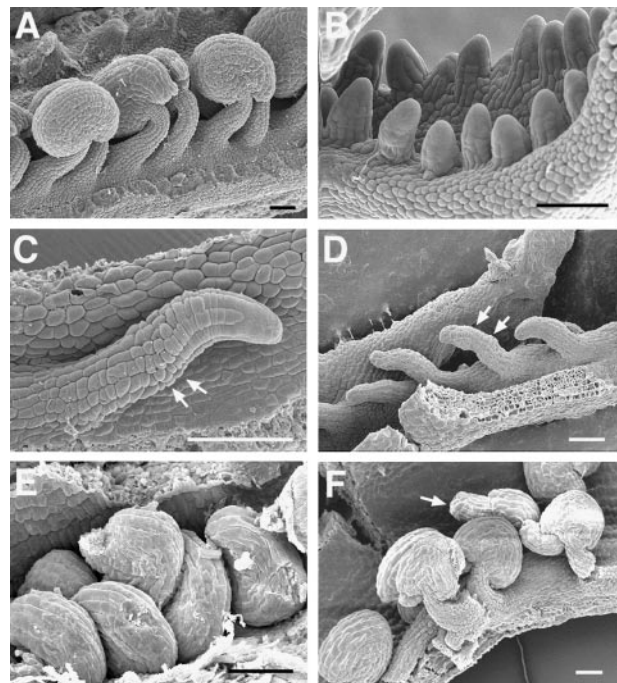
absence of normal interaction, as in the case of the *lug* single mutants, hornlike protrusions may occur, and in the case of *ett*, the stigmatic tissue may grow on the outer surface of the gynoecium (Sessions and Zambryski, 1995).

### **LUG and ANT Can Function as Either Positive or Negative Regulators, Depending on the Tissue Context**

Our study indicates that *LUG* and *ANT* promote medial ridge and marginal tissue development. This is in contrast to their roles as negative regulators of *AG* and, hence, of carpel identity. Thus, *LUG* and *ANT* may function as either a positive or a negative regulator, depending on the tissue context. This notion is best illustrated for *LUG* in the study of *CRC* (Alvarez and Smyth, 1999; Bowman and Smyth, 1999). *CRC* controls several aspects of carpel development and encodes a zinc finger and a helix-loop-helix domain. *CRC* mRNA is expressed in two distinct domains in gynoecium: the abaxial epidermal cell layer, and four internal groups of cells adjacent to the placental tissue (Bowman and Smyth, 1999). *LUG* influences the pattern of *CRC* expression in two opposite ways—by repressing *CRC* in the outer whorls of the flower and by promoting *CRC* expression in the four internal expression domains of the gynoecium (Bowman and Smyth, 1999). Perhaps *LUG* could interact with different partners to affect transcription in opposite ways. Such a mechanism might modulate its activity differently in flowers versus gynoecia.

Because *LUG* promotes *CRC* internal domain expression (Bowman and Smyth, 1999), *CRC* is a candidate target gene of *LUG* involved in medial ridge development. Like *lug* mutations, the *crc-1* null mutation causes a reduction of ovules. However, *crc-2*, a *CRC* promoter mutation that specifically abolishes the abaxial epidermal expression but leaves the internal expression domains intact, does not produce fewer ovules (Bowman et al., 1999; Eshed et al., 1999). Hence, the internal *CRC* expression domain is likely to promote the production of ovules, and the reduction of ovules in *lug* mutants probably reflects the loss of *CRC* internal expression domains. Nevertheless, *CRC* cannot be the sole factor in mediating the effect of *lug* on medial ridge development because *crc ant* double mutants do not exhibit the same defect as *lug ant* in medial ridge development (Eshed et al., 1999). Thus, *LUG* must regulate other genes in addition to *CRC* that are critical for medial ridge development.

Additional genes that participate in the development of gynoecium marginal tissue include *SPT*, *TSL*, and *PERIANTHIA (PAN)*. *spt* single mutant carpels lack a transmitting tract and exhibit defects in the postgenital fusion of the septum, whereas *crc spt* double mutant gynoecia exhibit a decrease in stigmatic papillae, style, ovules, and a complete loss of the septal tissue (Alvarez and Smyth, 1999; Bowman et al., 1999). *lug tsl* and *pan tsl* double mutants have also been noted to exhibit reduced marginal tissues (Roe et al., 1997). Nevertheless, none of these double mutants exhibits



**Figure 6.** Ovule Formation in Double Mutants of Negative Regulators of *AG*.

(A) *ap2-2* ovules at anthesis.

(B) A close-up of a *lug-2 ap2-2* double mutant carpel. Although the carpel is unfused, two rows of ovule primordia have initiated.

(C) An *ant-9* single mutant ovule. The ovule does not develop any integument. Arrows indicate the anticipated integument initiation site.

(D) A close-up of the *ant-9 ap2-2* double mutant carpel. Like *ant-9* single mutants, *ap2-2 ant-9* double mutants form ovules. These ovules do not have any integument. Arrows indicate the anticipated integument initiation site.

(E) *clf-2* single mutant seeds.

(F) Developing ovules in *lug-8 clf-2* double mutants. Some of these ovules have a protruding inner integument (arrow), similar to ovules of *lug* single mutants (Roe et al., 1997; Schneitz et al., 1997).

Bars = 50  $\mu$ m.

a defect in the marginal tissue development that is as striking and as complete as the one observed in *lug ant* double mutants. Thus, *TSL*, *PAN*, *CRC*, and *SPT* may all participate in the regulation of the development of gynoecium marginal tissue, acting either downstream of or together with *LUG/ANT*. In addition, genes expressed specifically in the medial ridge or marginal tissues could be candidate targets of *LUG/ANT* regulation—including *AGL1/AGL5*, *AGL 11*, *SHOOTMERISTEMLESS (STM)*, and several class I knotted-like homeobox proteins (Hake et al., 1995; Rousley et al., 1995; Savidge et al., 1995; Flanagan et al., 1996; Long et al., 1996). In situ examination of the expression patterns of these candidate target genes in *lug ant* double mutants may help

confirm their function and shed light on the mechanism of *ANT/LUG* action in gynoecial marginal tissue development.

## METHODS

### Plant Growth and Genetics

Seeds (*Arabidopsis thaliana*) were sown in Metro-Mix 200 soil (E.C. Geiger, Harleysville, PA). Biological larvicide Gnatrol (Penn State Seeds, Dallas, PA) was added to the water used to moisten the soil before sowing. The planted seeds were incubated at 4 to 10°C for 3 days and then were placed under constant cool white fluorescent lights at 20°C; the plants were fertilized ~14 days after germination with Miracle-Gro plant food.

Because *lug*, *ant*, and *ap2* all map to chromosome 4, and because *ant-9* is completely sterile, the double mutants between *lug* and *ant* or between *lug* and *ap2* were constructed as follows. *lug-1* and *lug-3* single mutant carpels were each pollinated by the *ap2-2/+ ant-9* transheterozygote plants. The wild-type  $F_1$  progeny segregated in  $F_2$  as either 1/4 *lug*, 1/4 *ant-9*, and 1/2 wild type (class I) or 1/4 *lug*, 1/4 *ap2-2*, and 1/2 wild type (class II). Seeds from 80 individual *lug-1* mutants (class I) were collected and planted, and eight of these 80 *lug-1* individuals segregated as 1/4 *lug-1 ant-9* double mutants in  $F_3$ . Similarly, seeds of 25 *lug-3*  $F_2$  individuals (class I) were collected, and four of these 25 segregated as 1/4 *lug-3 ant-9* double mutants in  $F_3$ . The *lug-1 ap2-2* double mutants were obtained by screening a large number of  $F_2$  progeny of *lug-1/+ ap2-2*. The *ant-9 ap2-2/+ ap2-2* and the *ant-9/+ ap2-2* seeds were kindly provided by Dr. David Smyth, and the *lug-8 clf-2* seeds were kindly provided by Dr. Justin Goodrich. In our experiments, *ant-9* mutants exhibited a less severe floral defect than previously reported in *ant-9* of the C24 ecotype (Elliott et al., 1996), possibly because of the Landsberg *erecta* (*Ler*) background introduced into the *ant-9* used in our studies.

### Microscopic Analyses

For scanning electron microscopy, the carpels, siliques, and flowers were first dissected under a stereo microscope and then fixed, coated, and photographed as described previously (Bowman et al., 1989, 1991a). Images were directly captured with the SEMICAPS software (SEMICAPS Inc., Santa Clara, CA) and the AMRAY 1000A scanning electron microscope. For the light microscopy histological sections, samples were fixed, stained, and sectioned using the in situ protocol (Drews et al., 1991). Tissues in 8- $\mu$ m-thick sections were dewaxed in 100% xylene, hydrated in ethanol series, and stained with toluidine blue. The stained tissues were dehydrated and mounted with Cytoseal 60 mounting medium (Stephens Scientific, Riverdale, NJ) and visualized and photographed under a Zeiss Axioplan2 microscope. For confocal microscopy, tissues were fixed, stained, and dissected according to Running et al. (1995) and were visualized with a Bio-Rad MRC 1024 confocal microscope.

### In Situ Hybridization

The floral tissue (entire inflorescence) was fixed as previously described (Drews et al., 1991), except that fixation time was 1 hr. The antisense *AG* probe was transcribed from the plasmid pCIT656

(Yanofsky et al., 1990). The antisense probe was synthesized using the Epicenter Technologies AmpliScribe T7 transcription kit (Madison, WI) according to manufacturer's protocol except that 5  $\mu$ L of 10 mM digoxigenin-11-UTP and cold UTP were added in 1:1 ratio. The probe was hybridized to the 8- $\mu$ m-thick sections of the tissue, which were then processed essentially as described by Lincoln et al. (1994) and Long and Barton (1998).

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#### NOTE ADDED IN PROOF

For the molecular analyses of *LEUNIG*, see J. Conner and Z. Liu, Proceedings of the National Academy of Sciences of the United States of America, volume 97, in press.

# Regulation of Gynoecium Marginal Tissue Formation by *LEUNIG* and *AINTEGUMENTA*

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