Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 5761–5765, June 1994
Plant Biology

Arabidopsis floral homeotic gene BELL (*BEL1*) controls ovule development through negative regulation of AGAMOUS gene (*AG*)

(flower development/pistil/carpel/seed/organ identity)

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Communicated by Paul K. Stumpf, February 18, 1994 (received for review February 4, 1994)

ABSTRACT **Ovules are the developmental precursors of seeds. In angiosperms the ovules are enclosed within the central floral organs, the carpels. We have identified a homeotic mutation in *Arabidopsis*, "bell" (*bell*), which causes transformation of ovule integuments into carpels. *In situ* hybridization analysis shows that this mutation leads to increased expression of the carpel-determining homeotic gene AGAMOUS (*AG*) in the mutant ovules. Introduction of a constitutively expressed *AG* transgene into wild-type plants causes the ovules to resemble those of *bell* mutants. We propose that the *BEL1* gene product directs normal integument development, in part by suppressing *AG* expression in this structure. Our results allow expansion of the current model of floral organ identity to include regulation of ovule integument identity.**

A typical angiosperm flower includes four major organ types—sepals, petals, stamens, and carpels. The carpels include an additional set of distinct internal structures, the ovules. As the precursors of seeds, ovules play an essential role in higher plant sexual reproduction. Ovules are also found in gymnosperms, which lack carpels, and fossil evidence of angiosperm ancestors demonstrates that ovules precede the evolution of carpels (1–3). Thus, ovules evolved first, with subsequent evolution of the enclosing carpel. From this perspective, ovules can be viewed as separate floral organs, which, in angiosperms, develop from and are enclosed within the carpels.

Little is known concerning the genetic control of determination and differentiation of ovules. This contrasts with recent progress in elucidation of control of development of other floral organs, where analysis of floral homeotic mutants in *Arabidopsis thaliana* and *Antirrhinum majus* has led to the formulation of two similar models for genetic determination of the identity of the four major floral organs (4–8). In *Arabidopsis*, at least five genes—APETALA1 (*AP1*), APETALA2 (*AP2*), APETALA3 (*AP3*), PISTILLATA (*PI*), and AGAMOUS (*AG*)—are responsible for this determination (5, 8–11). Several of these genes have now been cloned and found to be homologous to known transcription factors identified in yeast and mammals (12–15). One of these genes, *AG*, important for carpel identity (5, 8, 16), is also potentially involved in ovule development. The expression of *AG* occurs both early and late in flower development (16). Early *AG* expression, uniform in the carpel, determines the identity of this organ, whereas its late expression, restricted to subcompartments of carpels and ovules, may regulate specific cell fates (16). Two other *Arabidopsis* genes, *BEL1* and *SIN1*, mutations of which lead to aberrant ovules (17), specifically control ovule development. Here we show that several new *bell* alleles cause the homeotic transformation of ovule integuments into carpels, that this transformation is associated with overexpression of *AG* in these tissues, and that ectopic expression of *AG* from an introduced transgene can produce the same homeotic transformation. We propose an extension of an existing model of genetic control of floral organ identity to include *BEL1* as a negative regulator of *AG* within ovules.

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MATERIALS AND METHODS

Plant Material and Genetic Analysis. *Arabidopsis* ecotypes Landsberg *erecta* (Ler) and Columbia (Co-3) were obtained from Lehle Seed (Tucson, AZ), and No-0 was a gift from John Harada (Univ. of California, Davis). Plants were grown as described (18). Additional alleles of *bell* were isolated as described for *bell-1* (17). Allelism was established by complementation tests. All *bell* alleles were recessive to wild type, indicating that they probably represent loss-of-function mutations. Linkage of *bell* to *ttg* was deduced from segregation ratios in 246 F_2 progeny plants derived from crosses with strains having multiple markers (19) using the MAPMAKER computer program (20). Mapping relative to RFLP (restriction fragment length polymorphism) (21) was performed on F_2 progeny (96 chromosomes analyzed) from a *bell-1/bell-1* Ler × Co-3 cross. RFLP probes were M247 (*Bgl* II digestions) and g4028 (*Hin*dIII digestions). To determine the frequency of integument-to-carpel transformation in different alleles, ovules in >150 pistils from at least three different plants for each allele were scored for carpelloid features.

Microscopy and *in Situ* Hybridization. Scanning electron microscopy was performed as described (17). *In situ* hybridization was carried out as described earlier (22). ^{35}S-labeled sense and antisense RNA probes ($\approx 10^9$ cpm/μg) were made from AG cDNA clone pCIT565 as described (15). Probing with sense RNA gave uniformly low nonspecific background of silver grains. For measurement of surface density of autoradiographic grains, the images were digitized off the microscope and quantitated by using the IMAGE 1 image processor program. The grain surface density was total grain area divided by the total selected area. Wild-type and *bell-1* ovules obtained from late stage 14 flowers (16) were treated in the same experiment. Only exposures providing subsaturated grains were counted. For wild-type ovules, the area delimited by the endothelium and the embryo sac was not counted. The mean grain surface density (±SD) before background correction in the integument area on sections of wild-type ovules probed with anti-AG was 0.029 (±0.007) (10 ovules counted), and that for *bell-1* ovules with the same probe was 0.076 (±0.046) (13 ovules counted). The difference

Abbreviation: RFLP, restriction fragment length polymorphism.

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between the two means is highly significant (Student's t test $= -3.134$ for unequal variance; $P = 0.005$) (23). The mean background grain density was 0.02 ± 0.001, and the corrected means differed by a factor of >6.

Plant Transformation. Transformation of *Arabidopsis* ecotype No-0 was performed as described (24) with the Ti plasmid p35S-BAG (25). Seed was collected from 10 independent transgenic plants. Two of the resulting lines (TA4 and TA10) included plants that displayed the Bell$^-$ phenotype but were otherwise normal.

RESULTS

Integuments Are Converted to Carpels in *bell* Mutants. A mature wild-type flower of *Arabidopsis thaliana* contains approximately 50 ovules within the bicarpelloid gynoecium. Ovules are initiated as small finger-like primordia from the internal surface of carpels (the placentas) (17). An inner and an outer integument arise from the surface of each primordium, their site of origin demarcating the boundary between the apical nucellus and the supporting stalk (funiculus) of the ovule. The integuments grow to cover the nucellus and meet at the tip, leaving a small opening (the micropyle) (Fig. 1*A*). Within the nucellus a megasporocyte differentiates and undergoes meiosis, and one meiotic product develops into a seven-celled megagametophyte (26, 27). The nucellus then degenerates, and the innermost cell layer of the integuments differentiates into the endothelium that surrounds the embryo sac (17). After fertilization the ovule will develop into a seed, with the integuments forming the seed coat.

Plants homozygous for mutations in *bell* exhibit normal gross morphology but are female-sterile as a result of aberrant ovule development (17). In ovules of the original *bell* mutant (*bell-1*), a structure that initially resembles the outer integument initiates but develops abnormally into a collar-like structure, producing the characteristic bell-shaped ovules (17) (Fig. 1*B*). There is no evidence of formation of a separate inner integument. Meiosis occurs in the nucellus, but a normal embryo sac is not formed (17). Most *bell-1* ovules do not develop further and degenerate. We have mapped *bell* to chromosome 5 at 21.5 centimorgans (cM) from *ttg*, and 3.2 cM and 13.3 cM from RFLP markers m247 and g4028, respectively (with the order *ttg*, *bell*, m247, g4028).

We isolated five additional alleles of *bell*, which allowed us to define the critical role of the wild-type *BEL1* gene in controlling ovule development. In nearly every pistil of plants homozygous for *bell-4*, *-5*, or *-6*, one or more ovules develop

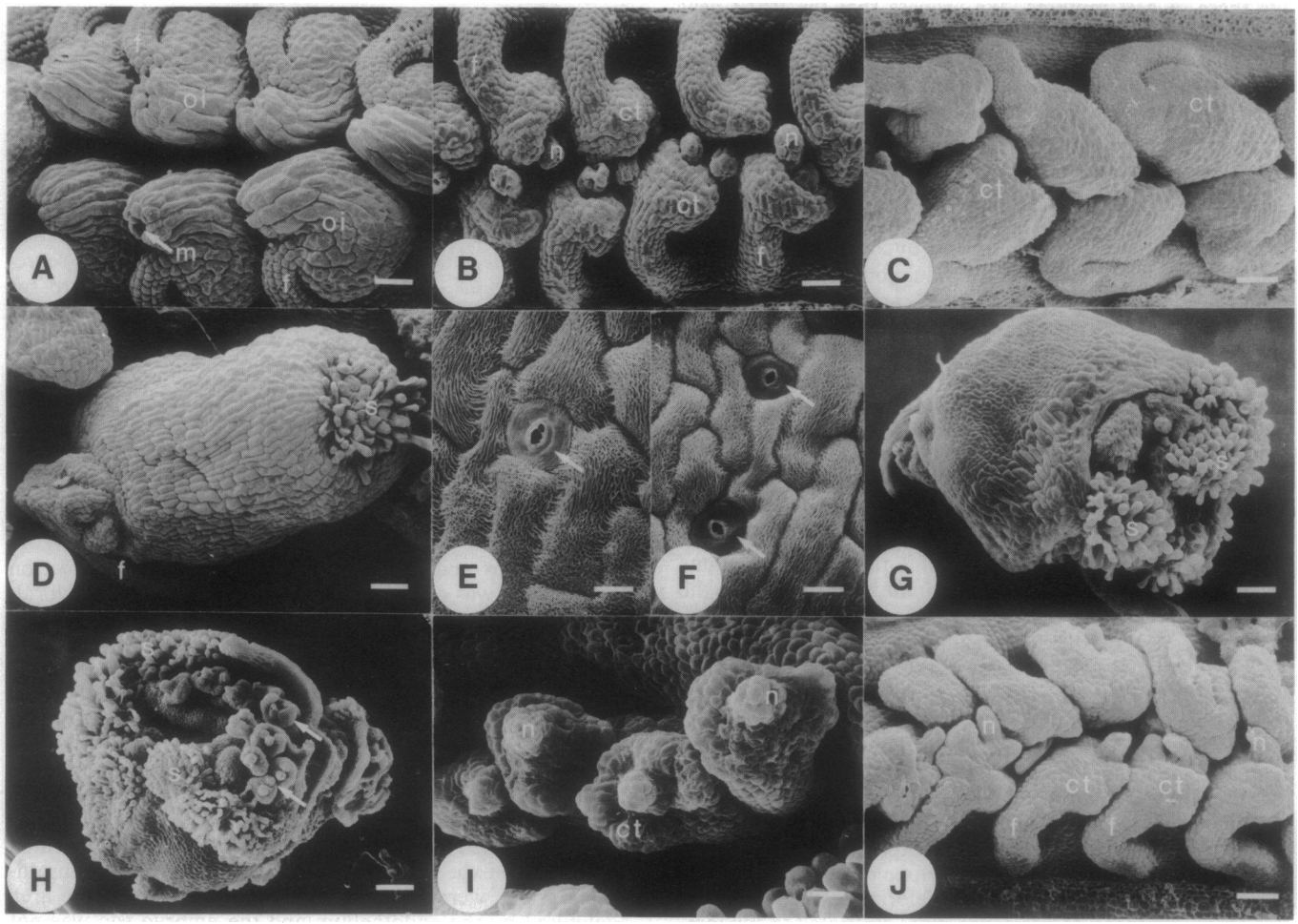

FIG. 1. Scanning electron micrographs of wild-type and mutant flower parts. (*A*) Ovules of wild-type plants at anthesis. The outer integuments (oi) cover the inner integuments and nucelli (not visible). (Bar = 25 μm.) (*B*) Ovules of the *bell-1* mutant at anthesis. The nucellus (n) is exposed, and the integuments are replaced by a collar of tissue (ct). (Bar = 30 μm.) (*C*) Ovules of the *bell-4* mutant at anthesis. The collars of tissue cover the nucelli but do not resemble wild-type integuments. (Bar = 40 μm.) (*D*) Ectopic carpel deriving from an ovule of a *bell-4* mutant. A stigma (s) has formed where the carpel has closed. (Bar = 50 μm.) (*E*) Epidermis of stylar region of carpelloid ovule of *bell-4* mutant. Arrow indicates stomate (Bar = 10 μm.) (*F*) Epidermis of stylar region of wild-type carpel. Arrows indicate stomates (Bar = 10 μm.) (*G*) Unclosed ectopic carpel of *bell-4* mutant. (Bar = 70 μm.) (*H*) Unclosed ectopic carpel of *bell-4* mutant showing secondary ovules (arrows). (Bar = 90 μm.) (*I*) Detail of ovules shown in *H*. (Bar = 20 μm.) (*J*) Ovules of transgenic plant containing the 35S-*AG* expression vector. (Bar = 30 μm.) m, Micropyle; f, funiculus.

into structures that resemble carpels. In these ovules the collar of tissue that replaces the integuments elongates to cover the nucellus (Fig. 1*C*) and then develops into a carpel-like cylinder with stigmatoid papillae at the apex (Fig. 1*D*). The exterior surface of the cylinder differentiates into an epidermis with characteristic cell shape, surface features, and stomata of the normal carpel outer epidermis (Fig. 1 *E* and *F*). The length of the entire structure is variable as is the position of the transformed *bell* ovules. In some cases the structure does not fully close but still develops the features of a normal carpel (Fig. 1*G*). The phenotype of *bell* mutants is iterative, with secondary ovules that display the Bel1$^-$ phenotype forming on the interior surface of the carpelloid ovules (Fig. 1 *H* and *I*). We conclude that the integuments are converted into carpels in this subset of *bell* ovules.

The frequency of fully carpelloid ovules shows an allelic series from fewer than one per 2500 ovules in the weakest alleles (*bell-1, -7, -8*) to an average of one in eight ovules in the most severe (*bell-5*). Even in the weakest alleles there is evidence for partial conversion of the ovules to carpels. In approximately 15% of the bell-shaped ovules in *bell-1* mutants, the nucellus contains a differentiated tracheary element (Fig. 2*A*) similar to elements present in wild-type carpels (Fig. 2*B*). Since vascular tissue in wild-type *Arabidopsis* ovules normally extends only slightly above the funiculus, these ectopic vascular elements may indicate partial conversion into carpelloid tissue. However, it is yet unclear if the nucellus also participates in the formation of the ectopic carpel structures that derive primarily from the integuments.

***bell* Ovules Ectopically Express *AG*.** The correlation between the site of *AG* expression and the eventual appearance of carpels or carpelloid structures (15, 16, 22) led us to postulate that aberrant *AG* expression could be responsible for the homeotic transformation of *bell* mutant ovules. We tested this hypothesis by *in situ* hybridization of sections of ovules with ^{35}S-labeled anti-AG cDNA (Fig. 2 *C–F*). In late stage 14 (after anthesis) wild-type ovules, *AG* expression is high in the endothelial cells and low in other integument layers (Fig. 2 *C* and *D*), confirming the results of Bowman *et al.* (16). By contrast, a significant level of AG message is found distributed uniformly within the *bell-1* ovules in flowers at this same developmental stage (Fig. 2 *E* and *F*). We quantitated the *in situ* hybridization signal (the number of autoradiographic grains per unit area) on sections of several wild-type and *bell-1* ovules. The level of AG mRNA in the collar of tissue that replaces the integuments in mutant ovules was significantly higher than that observed in the wild-type integuments, and this difference was even more pronounced when corrected for background. We conclude that the cells in the collar of tissue that replaces the integuments in a mature *bell* ovule actively express *AG* and propose that one role of *BEL1* is to suppress AG expression directly or indirectly in developing integuments.

Ectopic *AG* Expression Results in a Phenocopy of the *bell* Mutant. While the above experiments demonstrate an association between the *bell* mutation and ectopic expression of *AG* in the integuments, they do not imply a causal relationship between this expression and the homeotic transformation of the ovules. The role of *AG* expression in the Bel1$^-$ phenotype can be directly tested through induced overexpression of *AG* in otherwise wild-type plants. A T-DNA construct in which expression of the *Brassica napus* homolog of *AG* is driven by the strong "constitutive" 35S promoter of cauliflower mosaic virus (25) was introduced into wild-type

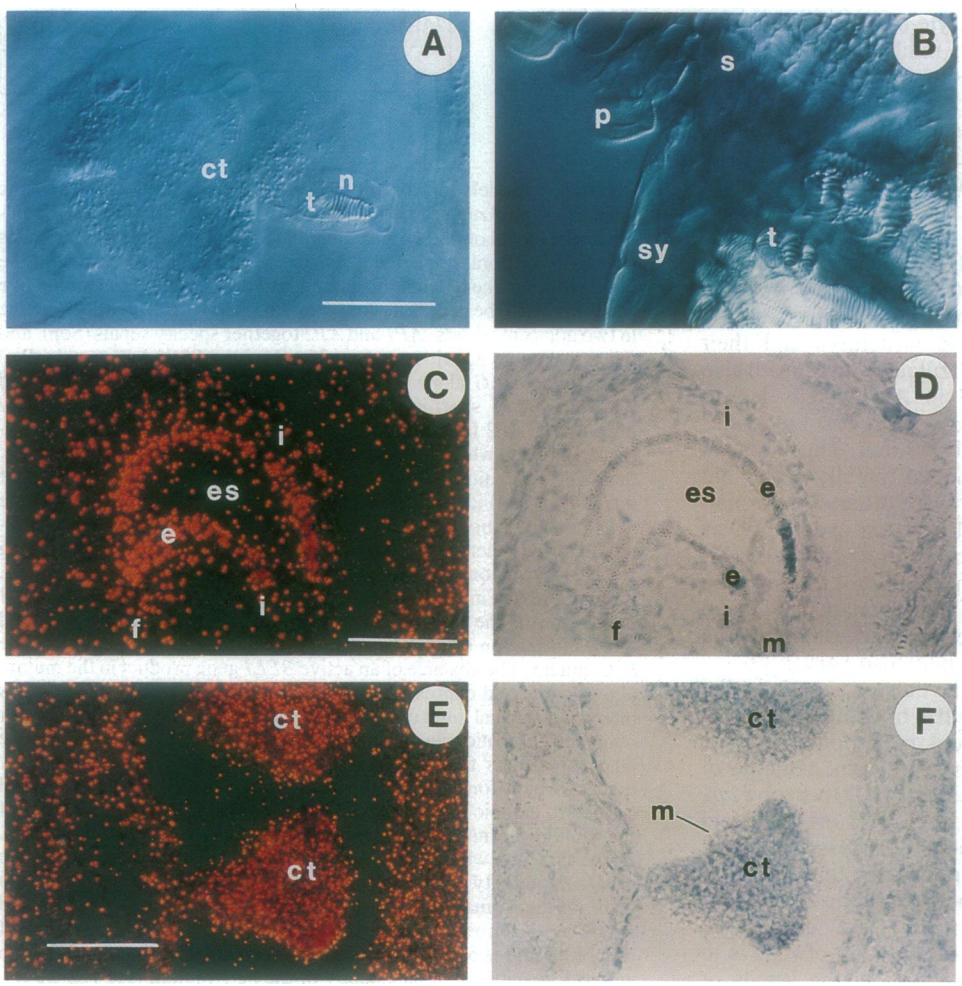

FIG. 2. Cleared whole mount and *in situ* hybridization analysis of *bell-1* ovules. (*A*) A cleared ovule of *bell-1* mutant showing differentiated tracheary element (t) in the nucellus (n) that projects out of the collar of tissue (ct) forming the main body of the mutant ovule. (*B*) Cleared stylar region (sy) of a wild-type pistil. Tracheary elements occur in profusion below the stigmatic papillae (s) with germinated pollen grains (p). Ovules and carpel were cleared (28) and photographed under Nomarski optics. (*C*) Section of a wild-type ovule at anthesis hybridized with the *AG* antisense probe. Orange grains represent hybridization signals. Most AG mRNA is seen in the single-cell layered endothelium (e) surrounding the embryo sac (es). Two integumentary layers (*i*) that surround the endothelium show much lower levels of hybridization. The funiculus (f) is attached at the base of the ovule. (*D*) Bright-field view of the same section as in *C*. The micropylar end of the ovule (m) does not have an endothelial lining. (*E*) Section through *bell-1* mutant ovules at anthesis hybridized with *AG* antisense probe. Hybridization signal is uniformly high over the collar of tissue (ct). (*F*) Bright-field view of the same section as in *E*. (Bars = 42 μm in *A* and *B*; 48 μm in *C*, *D*, *E*, and *F*.)

Arabidopsis plants. While ovules of the primary transgenic lines were wild type, progeny of two independent lines produced only bell-shaped ovules (Fig. 1*J*) or ovules in which the integuments had undergone a complete conversion to carpels (data not shown). The transgenic plants were normal in all other respects. Thus, *AG* overexpression is sufficient to duplicate the effects of loss of *BEL1* gene function, producing an exact phenocopy of the *bel1* mutant.

DISCUSSION

In summary, the above results show: (*i*) The integuments of some ovules in *bel1* plants are homeotically converted to carpels; (*ii*) the average level of AG mRNA in *bel1* ovules is higher than that observed in wild-type ovule integuments; (*iii*) constitutive *AG* expression in transgenic plants causes the formation of phenotypically Bel1$^-$ ovules.

The simplest interpretation of these results is that *BEL1* is a regulatory gene that restricts late *AG* expression in the integuments and that *BEL1* controls ovule development via its negative regulation of *AG*. In this view, unrestricted *AG* expression in *bel1* mutant ovules or constitutive expression of an *AG* transgene in a transformant causes the conversion of ovule integuments into a carpel. It is possible that a complete conversion requires at least a threshold level of *AG* expression. Thus, not all ovules in *bel1* mutants are completely transformed, and the frequency of transformation varies among different *bel1* alleles with various levels of residual *BEL1* activity. That we could produce phenocopies of *bel1* mutants in transgenic *Arabidopsis*, using a constitutively expressed *AG* homologue is consistent with previous results in which the same construct was introduced into tobacco plants (25). A subset of ovules of these transgenic tobacco plants were converted to style-like structures with terminal stigmatic papillae (25), an indication of a partial ovule to carpel conversion. Thus, ectopic expression of *AG* appears to be sufficient to produce phenotypically Bel1$^-$ ovules in two different species. Because the ovules in the transgenic tobacco plants were similar to previously described "stigmatoid ovules" of two tobacco mutants *mgr3* and *mgr9* (25, 29), it is possible that these mutations also alter *AG* expression in the ovules.

In addition to having carpelloid ovules, the transgenic tobacco plants overexpressing *AG* (25) exhibited properties similar to *Arabidopsis* apetala2 (*ap2*) mutants in that their sepals were converted to carpelloid structures and their petals were converted to stamens. Mizukami and Ma (30) also found that overexpression of *AG* in *Arabidopsis* led to phenocopies of *ap2* mutants, but they report that the ovules of these plants were morphologically normal. These results contrast with ours in which bell-shaped or fully carpelloid ovules were observed in plants that were otherwise normal. However, in other experiments, the vector we used was capable of producing Ap2$^-$ phenocopies in *Arabidopsis* (M. Yanofsky, personal communication). The best explanation for these results is that independent transformants may have different levels of expression of the transgene, and that the tissue-specificity of this expression may also vary.

Our results allow an extension of the current genetic model of floral organ identity to include control of ovule integument identity (Fig. 3). In our extended model, the proposed role for *BEL1* in the ovule exactly parallels a previous example of negative regulation of *AG* in the sepal by *AP2*. In strong *ap2* mutants, the sepals are converted to carpels or carpelloid structures (5, 31), and *AG* is ectopically expressed in these structures (22). As noted above, transgenes that constitutively express *AG* can lead to phenocopies of the ap2 mutant (25, 30), besides exhibiting phenotypically Bel1$^-$ ovules (see above). It has been proposed that one function of AP2 is to negatively regulate the expression of *AG* in the sepals of

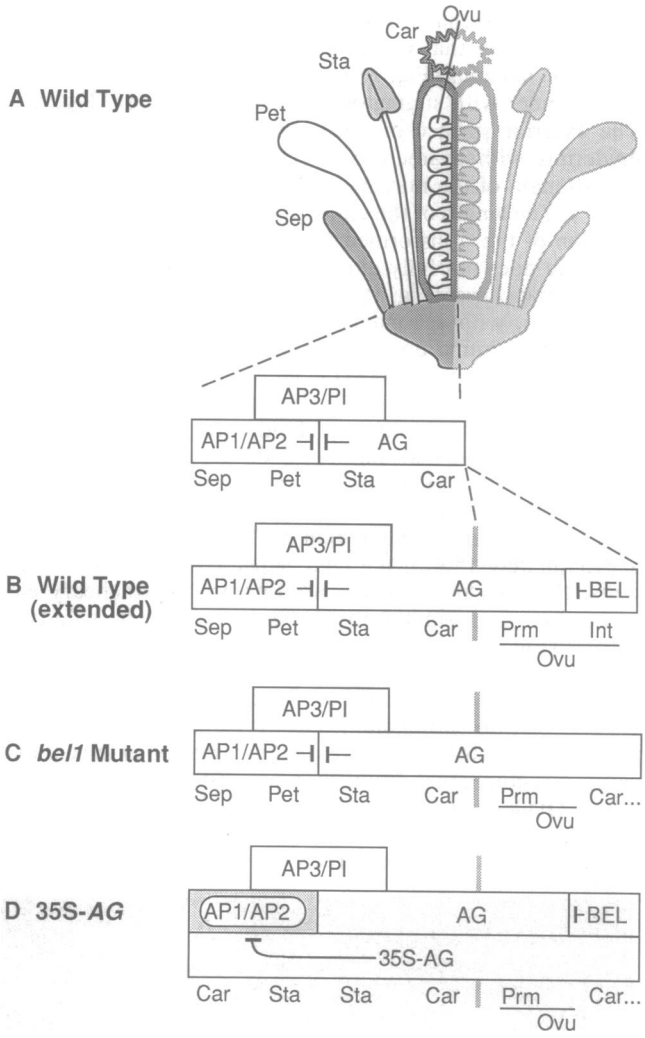

FIG. 3. Models for control of floral organ identity. (*A*) Schematic diagram of an *Arabidopsis* flower and the previously published combinatorial model of floral organ identity (5, 8, 9). Boxes represent zones of activity of floral homeotic genes in the four organ whorls, with the outer whorl on the left and the inner whorl on the right. Each of the five genes known to be necessary for specifying floral organ identity is active in two adjacent whorls. *AP1* and *AP2* together specify sepals (Sep), and when these two activities are combined with *AP3* and *PI*, petals (Pet) are specified. *AG* alone specifies carpels (Car) and, when combined with *AP3* and *PI*, specifies stamens (Sta). Expression of *AG* and activity of the *AP1/AP2* function are mutually antagonistic (T-bars). (*B*) Extended model, which includes aspects of ovule (Ovu) development, including formation of ovule primordia (Prm) and determination of integument (Int) identity. The shaded vertical bar indicates that ovule development is both spatially and temporally separated from determination of the other floral organs, taking place inside the carpels during stage 9 (17) and on the floral apex during stages 5–7 (10), respectively. *AG* is expressed in stamens, carpels, and in ovule primordia (16). *BEL1* expression in the integument primordia inhibits *AG* expression and directs formation of integuments. (*C*) Extended model applied to the *bel1* mutant. The absence of an active *BEL1* gene product in the mutant allows continued expression of *AG* in integument primordia. *AG* thus interrupts normal integument development and causes a partial or complete homeotic transformation of the integuments into a carpel. The ellipsis indicates that this process can be repeated in the secondary ovules, which form within the ectopic carpel. (*D*) Extended model applied to ectopic *AG* expression. Expression of *AG* under control of the cauliflower mosaic virus 35S promoter is in all parts of the flower. In the first two whorls, this ectopic *AG* expression can suppress the *AP1/AP2* activity (indicated by the shading within the box) and can result in the formation of carpels and stamens in place of sepals and petals as described (25, 30). Expression of *AG* in the integument primordia causes partial or complete conversion of the integuments into a carpel, resulting in a phenocopy of the *bel1* mutant (see Fig. 1*J*).

wild-type flowers (5) (Fig. 3*A*). By analogy, our extended model (Fig. 3*B*) explains the phenotypes observed in both *bell* mutants and in plants containing the constitutive *AG* transgene (Fig. 3 *C* and *D*) with the postulate that *BEL1* negatively regulates *AG*. Thus, *AG* expression in the flower may be controlled at several places by different regulators. *AG* expression is negatively regulated by *BEL1* in ovules, and by *AP2* in sepals. In addition, *AG* may be negatively regulated in anthers by an as yet unidentified gene because *AG* expression is restricted in this organ (16), and plants constitutively expressing *AG* can be male sterile (25, 30).

While more complex models wherein *bell* indirectly effects ovule-to-carpel transformation are possible, our proposal appears to be the simplest. Our observation that ovule integuments can be converted to a carpel by the loss of a single gene may have implications for theories on the evolutionary origins of integuments and carpels, a topic that remains controversial (1, 2, 32). In combination with ongoing research on the determination of major floral organs (4–8), further studies on *bell* and on additional mutations affecting ovule development will allow construction of increasingly predictive and accurate molecular-genetic models of flower and ovule ontogeny.

Note Added in Proof. Similar results concerning carpelloid ovules and ectopic expression of *AG* in *bell* mutants were recently reported (33).

We thank Elliot Meyerowitz for providing pCIT565; Martin Yanfosky for p35S-BAG; Detlef Weigel and Lynne Angerer for instructions on *in situ* hybridization; and Lynne Angerer, John Bowman, Judy Callis, Tom Eickbush, J. Christopher Gaiser, and J. Clark Lagarias for critical reviews of the manuscript. This work was supported by Cooperative State Research Service Awards, U.S. Department of Agriculture 92-37304-7966 (to A.R.) and 92-37304-7756 (to C.S.G.), U.S. Department of Agriculture National Needs Graduate Fellowship (to S.C.B.), and National Science Foundation Postdoctoral Fellowships in Plant Biology (to K.R.-B. and D.P.).

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