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SHORT COMMUNICATION

BELL1 and *AGAMOUS* genes promote ovule identity in *Arabidopsis thaliana*

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Summary

Molecular and genetic analyses have demonstrated that the *Arabidopsis thaliana* gene *BELL1* (*BEL1*) is required for proper morphogenesis of the ovule integuments. Several lines of evidence suggest that BEL1 may act, at least in part, to repress the function of the organ identity gene *AGAMOUS* (*AG*) during ovule development. To study the relative roles of *BEL1* and *AG*, plants homozygous for *ag*, *bel1* or both were constructed in an *ap2* mutant background where ovules form even in the absence of *AG* function. The loss of either *BEL1* or *AG* led to a decrease in the number of mature ovules, accompanied by an increase in primordial outgrowths. These data suggest that *BEL1* and *AG* gene products act early in ovule development in a partially redundant manner to direct ovule identity. Development of the abnormal integuments characteristic of the Bel1$^-$ mutant phenotype was found to be dependent on *AG* function. Finally, *BEL1* appears to be required for embryo sac development independent of both other aspects of ovule morphogenesis and *AG* function. This study therefore suggests that both BEL1 and AG are required for several distinct aspects of ovule morphogenesis.

Introduction

Many *Arabidopsis* genes involved in ovule development have been identified, including *BELL1* (*BEL1*) (reviewed in Gasser *et al.*, 1998). *BEL1* encodes a putative homeodomain transcription factor that is expressed throughout the young ovule primordium and becomes limited to the central region prior to the initiation of the integuments. Consistent with this expression pattern, loss-of-function mutations in *BEL1* result in abnormal ovules (Bel1$^-$ ovules) that lack an inner integument, have an irregular-shaped lateral structure in place of the outer integument, and rarely complete megagametogenesis (Modrusan *et al.*, 1994; Robinson-Beers *et al.*, 1992).

Several lines of evidence suggest that *BEL1* is an ovule-specific negative regulator of *AGAMOUS* (*AG*), the floral organ identity gene required for the development of reproductive organs (Modrusan *et al.*, 1994; Ray *et al.*, 1994; Reiser *et al.*, 1995). First, after anthesis, the abnormal outgrowth of some Bel1$^-$ ovules forms a structure resembling a carpel, complete with secondary ovules. Second, there is persistent expression of *AG* mRNA throughout Bel1$^-$ ovules at a time when it is normally restricted to a single cell layer. Finally, over-expression of *Brassica napus AG* in *Arabidopsis* resulted in Bel1$^-$-like ovules. While the mechanism by which *BEL1* influences *AG* in the ovule is not known, evidence indicates that it acts post-transcriptionally (Reiser *et al.*, 1995). Whether *BEL1* is needed primarily to regulate *AG* function or is required more directly for ovule development remains to be determined.

It is unclear whether *AG* itself has a specific role in wild-type ovule development. *AG* is transcribed in the ovule in a complex temporal and spatial pattern (Bowman *et al.*, 1991a; Reiser *et al.*, 1995), suggesting that it may be involved in several aspects of ovule morphogenesis. *Ag-1* mutants lack carpels and do not make associated ovules. Flowers doubly mutant for *AG* and another floral organ identity gene, *APETALA2* (*AP2*), however, have mosaic, carpel-like sepals which bear ovules (Bowman *et al.*, 1991b). Some of these *ap2 ag* ovules have wild-type morphology, suggesting that *AG* is not required for ovule development.

To clarify the roles of *BEL1* and *AG* during ovule development, we undertook a detailed analysis of the ovule phenotypes of *ag*, *bel1* and *ag bel1* in an *ap2* background. Our data suggest that both *BEL1* and *AG* are required for multiple aspects of ovule development, including ovule identity and integument morphogenesis.

Results

To determine the relative roles of *AG* and *BEL1* during ovule development, the ovule phenotypes of *ap2 ag*, *ap2 bel1* and *ap2 ag bel1* mutant flowers were examined (Figure 1). To control for differences in background, two similar sets of double and triple mutants were constructed differing in the *ap2* and *bel1* alleles used (*ap2–6*, *bel1–3*, *ag-1* versus *ap2–2*, *bel1–1*, *ag-1*). Since similar results were

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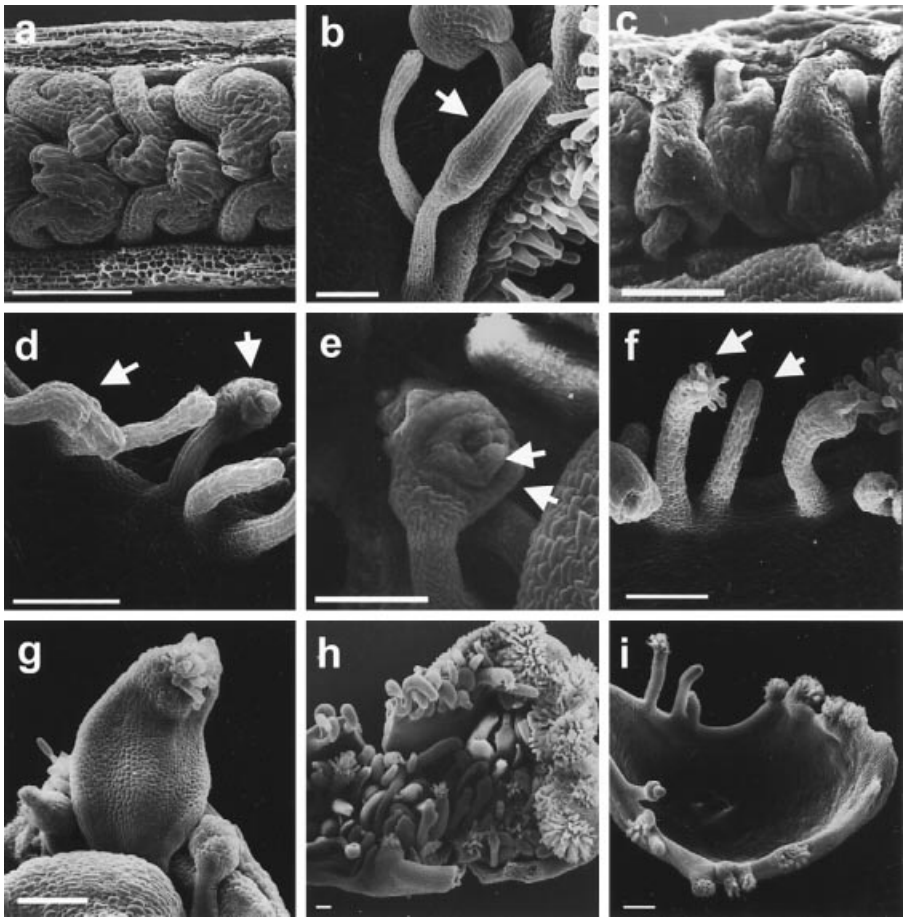

Figure 1. Scanning electron micrographs of the ovule types found in *ap2–6*, *ap2–6 bel1–3*, *ap2–6 ag-1* and *ap2–6 ag-1 bel1–3* mutant flowers.
Magnification bars equal 100 μm. (a) Wild-type ovules. (b) A Flo10^{-}-like ovule (arrow) on an *ap2–6 ag-1* double mutant carpel-sepal. (c) Bel1^{-} ovules in an *ap2–6 bel1–3* double mutant gynoecium. (d) Immature ovules (arrows) on an *ap2–6 ag-1* double mutant carpel-sepal. (e) Immature ovule on an *ap2–6 bel1–3* double mutant carpel sepal. Note that two integuments (arrows) are present on only one side of the nucellus. (f) Projection ovules on a carpel sepal of an *ap2–6 ag-1* double mutant heterozygous for *BEL1/bel1–3* (arrows). (g) Planar ovule on an *ap2–6 bel1–3* double mutant carpel-sepal. (h) A typical *ap2–6* mutant carpel-sepal. Note the variety of structures. (i) A typical *ap2–6 ag-1 bel1–3* triple mutant carpel-sepal.

observed for both sets of mutants, we will describe in detail only those for one set (*ap2–6*, *bel1–3*, *ag-1*). The ovule phenotypes found in each of these mutant lines were extremely variable, and we classified them based on their gross morphology into four basic categories (Figure 1 and Table 1).

Mutant ovule morphology

Mature ovules. Ovules with well-developed integuments were termed mature ovules. Included in this category are ovules with a wild-type morphology (Figure 1a) and ovules with symmetrical integuments resembling those of *flo10* (*superman*) mutant flowers (Figure 1b; Gaiser *et al.*, 1995).

Bel1^{-} ovules. All ovules lacking an inner integument and having an abnormal lateral structure in place of the outer integument, similar to the mature ovules of *bel1* mutant flowers, were categorised as Bel1^{-} ovules (Figure 1c).

Immature ovules. Structures which, when observed by SEM, resemble ovules with one or two integuments arrested at various developmental stages were classified as immature ovules (Figure 1d,e).

Primordial outgrowths. Structures that had no ovule features but arose from the placental tissue fell into two groups. Finger-like projections of cells were termed projection ovules (Figure 1f). These could be short with domed tips similar to ovule primordia or longer, bearing stigmatic papillae. Large, flat, leaf-like structures that sometimes had stigmatic papillae at their tips (Figure 1g) were termed planar ovules. Examination by SEM showed that planar ovules were also composed of ovary-like cells, suggesting a carpel character.

The ovules of ap2–6 *mutants*

The flowers of strong *ap2* mutants (e.g. *ap2–6*, *ap2–2*) have carpel-sepal organs in the outer whorls, followed by stamens or stamen-carpel organs, and a bicarpellate gynoecium that is sometimes unfused. *ap2* outer whorl carpel-sepals are marked by stigmatic papillae and placental tissue bearing structures resembling ovules (Figure 1h; Bowman *et al.*, 1991b; Kunst *et al.*, 1989).

We studied the ovules of *ap2–6* gynoecia, as well as ovules on the carpel-sepals, as a basis for comparison with the double and triple mutant phenotypes. The majority of structures in *ap2–6* gynoecia were mature, morphologically

Table 1a. Percentage of ovule types found in the gynoecia of *ap2–6* and *ap2–6 bel1–3*[a]

Mutant	Number of flowers	Ovule type					
		Mature		Bel1⁻	Immature	Primordial outgrowths	
		Wild type	Flo 10			Projection	Planar
ap2–6[b]	172	94 (11 715)	1 (97)	0	0	< 1 (31)	4 (541)
ap2–6 bel1–3[c]	23	0	0	70 (460)	26 (173)	0	4 (25)

[a]Number of ovules are in parentheses.
[b]Dissection microscope, includes both locules.
[c]SEM data, one locule per flower.

Table 1b. Percentage of ovule types found on the carpel-sepals of mutants[a,b]

Mutant	Number of flowers	Ovule type					
		Mature		Bel1⁻	Immature	Primordial outgrowths	
		Wild type	Flo 10			Projection	Planar
ap2–6	34	49 (650)	6 (81)	0	5 (66)	16 (209)	24 (320)
ap2–6 bel1–3	59	0	0	6 (95)	31 (470)	30 (449)	32 (484)
ap2–6 ag-1[c]	63	33 (618)	3 (68)	0	6 (119)	54 (1021)	3 (63)
ap2–6 ag-1 bel1–3	70	1 (20)	0	0 (1)	11 (272)	76 (1832)	12 (300)

[a]SEM data.
[b]Number of ovules are in parentheses.
[c]Isolated from the triple-segregating population.

normal ovules (95%), accompanied by small numbers of primordial outgrowths (5%) (Table 1a).

The ovules of the carpel-sepals were much more variable (Figure 1h and Table 1). The majority of ovules were either mature (55%) or primordial outgrowths (40%), with only a small proportion of immature ovules (5%) (Table 1b). In addition, there were fewer ovules than found in each carpel of the gynoecium (an average of 22 per carpel-sepal versus 36 per locule of the gynoecium). To confirm that the mature ovules seen on carpel-sepals were histologically normal, ovules were cleared. The majority had both inner and outer integuments and embryo sacs (94%) (Figure 2a), while a few had underdeveloped embryo sacs (6%) (16 ovules counted). Clearing of several (5) immature ovules revealed that most had a megaspore or developing embryo sac (4/5) while one (1/5) did not.

The ovules of ap2–6 bel1–3 *double mutants*

ap2–6 bel1–3 double mutant flowers resemble *ap2–6* mutant flowers with Bel1⁻ ovules (Figure 1c) (Modrusan *et al.*, 1994). To determine the role of *BEL1* function we analysed the ovule types in these flowers in more detail (Table 1) and compared the results to those of the *ap2–6* mutant. In the *ap2–6 bel1–3* gynoecium, there were 70% Bel1⁻, 25% immature, and 4% primordial outgrowths

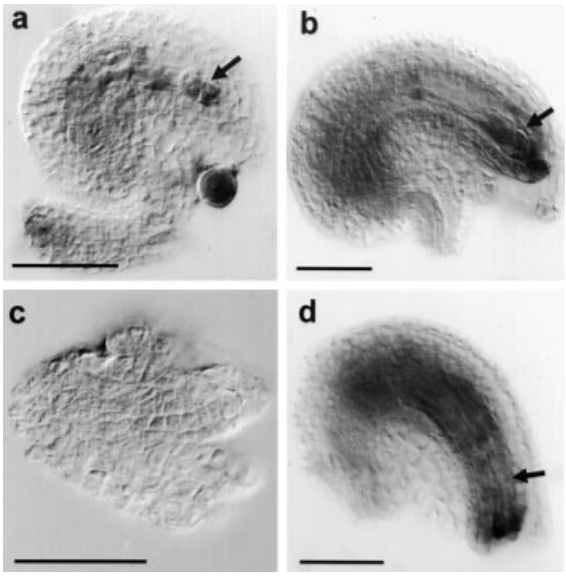

Figure 2. Wild-type and mutant ovules cleared in organic solvents and photographed under differential interference contrast optics.
Magnification bars equal 50 μm.
(a) Wild-type ovule with distinct embryo sac (arrow).
(b) Mature ovule from *ap2–6 ag-1* double mutant leaf-carpel with distinct embryo sac (arrow).
(c) *bel1–3* mutant ovule. Note the lack of an embryo sac.
(d) Mature ovule from *ap2–6 ag-1 bel1–3* mutant leaf-carpel. Note the space with files of cells where the embryo sac should be (arrow).

(Table 1a). Relative to *ap2–6* mutants, the *ap2–6 bel1–3* double mutant gynoecia had a lower percentage of mature (Bel1$^-$) ovules and a higher percentage of immature ovules (Table 1a). Interestingly, the gynoecial immature ovules were seen in *ap2–6 bel1–3* but not in *ap2–2 bel1–1* mutants, suggesting that this aspect of the phenotype may be due to allele or background specific effects.

ap2–6 bel1–3 mutant carpel-sepals had 6% Bel1$^-$, 31% immature, and 62% primordial outgrowths (Table 1b). A comparison with *ap2–6* shows that, as in the gynoecium, the loss of *BEL1* function results in the presence of Bel1$^-$ and the absence of wild-type ovules. These data indicate that the *BEL1* gene is required for integument development, as has been suggested previously (Modrusan *et al.*, 1994; Robinson-Beers *et al.*, 1992).

The ovules of ap2–6 ag-1 *double mutants*

ap2–6 ag-1 double mutant flowers consist of leafy organs followed by two whorls of mosaic petal-stamen organs, then inner flowers which follow the same pattern. The outer whorl leafy organs, like Ap2$^-$ carpel-sepals, can have stigmatic papillae and placental tissue giving rise to structures resembling ovules (Figure 1i) (Bowman *et al.*, 1991b). To determine the role of *AG*, we examined the ovules of *ap2–6 ag-1* double mutant flowers and compared them to *ap2–6* gynoecia and carpel-sepals (Table 1). Carpel-sepals of the inner and outer whorls of *ap2–6 ag-1* mutant flowers were relatively indistinguishable from each other with respect to ovule number and type (data not shown), thus ovule data from all carpel-sepal organs were combined (Table 1b). All the ovule types seen on *ap2–6* outer whorls were also found on *ap2–6 ag-1* double mutant carpel-sepals (mature, immature and primordial outgrowths). Clearing confirmed that the mature ovules were histologically normal and that most mature and immature ovules contained embryo sacs (89%; 55 ovules examined) (Figure 2b). There were 6 times fewer ovules per *ap2–6 ag-1* carpel-sepal (average = 5) than observed for *ap2–6* gynoecia (average = 36/carpel) and four times fewer than observed for *ap2–6* mutant carpel-sepals (average = 22). This decrease suggests an effect on the initiation of ovule primordia.

In comparison to ovules of *ap2–6* mutant carpels or carpel-sepals, the proportion of *ap2–6 ag-1* ovules that are mature was significantly lower (36% in *ap2–6 ag-1* compared with 94% in *ap2–6* gynoecia or 55% in *ap2–6* carpel-sepals), while the proportion of primordial outgrowths was larger (57% in *ap2–6 ag-1* compared with 5% in *ap2–6* gynoecia or 40% in *ap2–6* carpel-sepals) (Table 1b). These data suggest that *AG* plays a role in the establishment of ovule identity following initiation of ovule primordia.

The ovules of ap2–6 ag-1 bel1–3 *triple mutants*

To further investigate the roles of *AG* and *BEL1* in ovule development and to determine if there are any interactions between these two genes, *ap2–6 ag-1 bel1–3* triple mutants were constructed and the ovule phenotype compared to that of the *ap2–6 bel1–3* and *ap2–6 ag-1* double mutants.

The triple mutants had all the ovule types found on *ap2–6* and *ap2–6 ag-1* mutant carpel-sepals (Table 1b). The majority of structures were primordial outgrowths (88%), with some immature ovules (11%) and some rare ovules with a wild-type morphology (20/2424 ovules counted). *ap2–6 ag-1 bel1–3* triple mutant ovules with a wild-type morphology were examined by clearing in organic solvents. The outer integument did not always grow to cover the inner integument to the same extent as seen in normal ovules. In addition, in 13/14 ovules with wild-type morphology, there was no sign of an embryo sac, merely a space bordered by the endothelium where the gametophyte would normally be found (Figure 2d), as seen for some mutants affected in megagametogenesis (Schneitz *et al.*, 1997). In the remaining ovule, the integuments surrounded what appeared to be a degrading mega-gametophyte. A similar trend was seen in the ovules with short integuments and in immature ovules, where 77/99 lacked an obvious mega-gametophyte. Unfortunately, ontological investigation to determine the exact nature of the embryo sac defect was impossible due to the inability to predict which of the developing ovules would reach maturity.

The ovule phenotypes of *ap2–6 ag-1 bel1–3* and *ap2–6 ag-1* mutant carpel-sepals were compared to yield information about the role of the *BEL1* gene. The proportion of mature ovules with a wild-type morphology was significantly lower in *ap2–6 ag-1 bel1–3* (1% – 20/2424) compared with *ap2–6 ag-1* mutants (36% – 686/1889). In addition, the percentage of primordial outgrowths was significantly higher (88% instead of 57%). The higher proportion of primordial outgrowths observed in *ap2–6 ag-1 bel1–3* mutants relative to *ap2–6 ag-1* mutants suggests that the BEL1 protein may also function early in development, in a partially redundant manner with the AG protein to establish ovule identity. The lack of an embryo sac in mature ovules of the *ap2–6 ag-1 bel1–3* triple mutant was similar to the phenotype of *bel1–3* and *ap2–6 bel1–3* mutant ovules (Figure 2c,d; Modrusan *et al.*, 1994) but unlike those of *ap2–6 ag-1* mutant mature ovules (Figure 2b), suggesting that *BEL1* but not *AG* gene function is required for normal embryo sac development.

The ovule phenotypes of ap2–6 ag-1 bel1–3 and ap2–6 bel1–3 mutant carpel-sepals were compared to yield information about the role of the *AG* gene. The ap2–6 bel1–3 double mutant had a significantly lower percentage of primordial outgrowths relative to the triple mutant (4%

on the carpels and 62% on the carpel-sepals compared with 88% on the ap2–6 ag-1 bel1–3 triple mutant carpel-sepals). These data are consistent with the idea that *AG* is required to establish ovule identity. Unlike the ap2–6 bel1–3 double mutants, ovules with mature Bel1⁻ morphology were not seen in the triple mutant, with one exception (1/2424 ovules counted, Table 1). Although immature ovules with a single integument were observed in the triple mutant (Figure 1i), they were phenotypically distinct from Bel1⁻ ovules as they differed in shape, extent of growth and/or position of the integument-like structure (compare Figure 1c with Figure 1i). In addition, single-integument ovules similar in morphology to those of the triple mutant were present in mutants where *BEL1* was still functional (ap2–6 and ap2–6 ag-1; see Figure 1d). The absence of Bel1⁻ ovules in the triple mutant was not simply due to an inability to form mature ovules in the absence of *AG* activity. Indeed, ovules with wild-type morphology were found in the triple mutant but not in the ap2–6 bel1–3 double mutant (Table 1 and Figure 2d). These data suggest that *AG* function is required for the Bel1⁻ phenotype.

Discussion

Previous research has suggested that the *BEL1* gene product acts to negatively regulate *AG* function during ovule development in *Arabidopsis*. We have studied the relative roles of these two genes using a genetic approach. Our results verify that *AG* is required for the Bel1⁻ ovule phenotype and that *BEL1* has an affect on mega-gametogenesis. Contrary to previous thought, however, our data also suggest that *AG* promotes the specification of ovule identity, a role that it shares in a redundant manner with *BEL1* and other, as yet unspecified, genes (Figure 3).

AG *has an active role during ovule development*

Beyond its role in determining carpel identity, *AG* has not been considered a player in ovule development since normal ovules can develop in its absence (Bowman *et al.*, 1991a) and its function can be detrimental to ovule development (Ray *et al.*, 1994). In contrast, our results suggest that AG functions to promote ovule identity (Figure 3).

Our analyses of *ap2* ovules relative to *ap2 ag* and *ap2 ag bel* mutant ovules showed significant decreases in mature wild-type ovules and an increase in planar and/or projection ovules. These primordial outgrowths, apart from their position on placental tissue, had no ovule features except for an enlarged cell resembling a megaspore in a few projection ovules. More frequently, the projection and planar ovules showed carpel characteristics including stigmatic papillae and ovary cells. Thus, the primordial outgrowths could be considered to be the result of a defect in the establishment of ovule identity following the initiation of primordia from the placenta (Figure 3). This hypothesis is consistent with the data and conclusions of Hicks and McHughen (1974) and Evans and Malmberg (1989) who cultured wild-type tobacco placentas at different stages of carpel development. Culturing placentas bearing ovule primordia that had not yet undergone ovule specific morphogenesis resulted in the primordia adopting a carpel-like fate. Analogous ovule phenotypes have been observed in the tobacco mutants Mgr3/9 (Evans and Malmberg, 1989), plants co-suppressed in two petunia MADS-box genes (*FBP7* and *FBP11*; Angenent *et al.*, 1995), and plants ectopically expressing *B. napus AG* in tobacco (Mandel *et al.*, 1992).

The role of *AG* function during ovule development is not

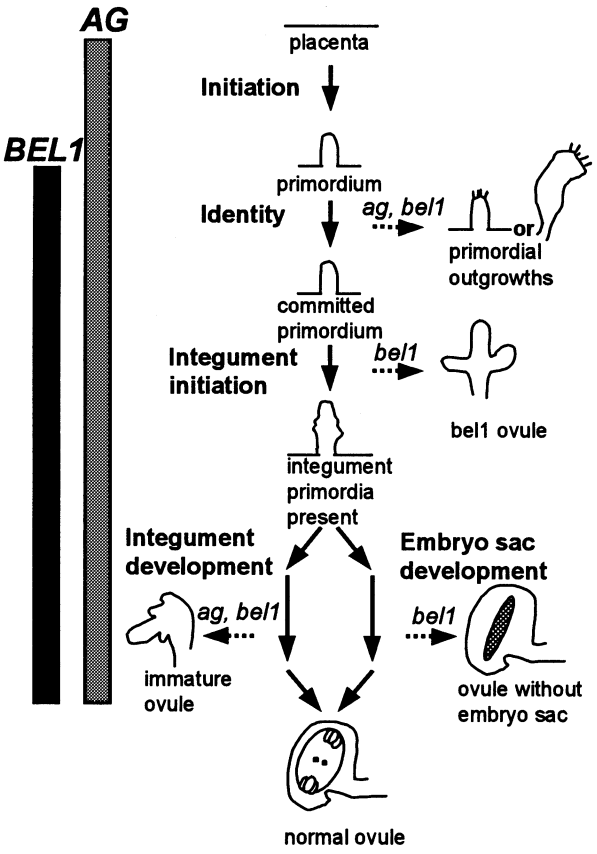

Figure 3. A model showing the timing and roles of *AG* and *BEL1* throughout ovule development and the effects of their absence.
The major stages of *Arabidopsis* ovule development are shown as a linear pathway from placenta to mature ovule. The processes occurring in between each of these stages are shown in bold. At the far left are bars showing the timing of mRNA expression of *AG* (grey) and *BEL1* (black). Our results suggest that *AG* is involved in ovule identity and possibly in integument development, while *BEL1* acts during the specification of ovule identity, integument morphogenesis and embryo sac development. *AG* appears to have a greater role early in development, whilst *BEL1* has a stronger role in the later stages. To the right and left of the developmental progression are diagrams of the various ovule types found when either *AG* and/or *BEL1* are mutated, placed at the stage of ovule development at which they deviate from the norm.

necessarily direct. Ovule defects observed in an *ap2 ag* background could be an indirect result of the plant's inability to develop a normal gynoecium. *AG* transcripts, however, are found in the placental tissue prior to ovule initiation (Bowman *et al.*, 1991a) and in the developing ovule primordium in a pattern similar to that of *BEL1* (Reiser *et al.*, 1995). The fact that this *AG* ovule specific expression pattern correlates with the ovule's defects associated with loss of AG function suggests that the roles of AG in ovule identity and integument morphogenesis are direct.

BEL1 *and* AG *act in parallel to control ovule identity*

In addition to roles in integument and embryo sac development, our results suggest that *BEL1*, like *AG*, is involved in the specification of ovule identity in the early stages of ovule development, a hypothesis supported by the presence of *BEL1* transcript throughout the newly initiated ovule primordium (Reiser *et al.*, 1995). This role could only be observed in the absence of *AG* function, suggesting that *AG* activity is sufficient to promote ovule identity even in the absence of *BEL1*. In contrast, the primordial outgrowths among *ap2–6 ag-1* double mutant ovules suggests that *BEL1* activity alone is insufficient to promote identity in all developing ovules.

A comparison of the ovule types found on *ap2–6* carpels and the *ap2–6 ag-1* and *ap2–6 ag-1 bel1–3* double and triple mutant carpel-sepals reveals an additive phenotype which suggests that *AG* and *BEL1* promote ovule identity independently of one another. Thus, with respect to ovule identity, *AG* and *BEL1* could be considered to function redundantly in parallel pathways. Partial functional redundancy has already been observed for genes regulating other aspects of ovule morphogenesis (Schneitz *et al.*, 1998).

Despite the large number of female sterile mutants of *Arabidopsis* isolated to date, no genes in addition to *AG* or *BEL1* have been identified as having roles in ovule identity. Additional genes involved in this process may have gone undetected because of functional redundancy (as in the case of *FBP7* and *FBP11* in petunia; Angenent *et al.*, 1995), or because they have roles in earlier developmental events (as in the case of *AG*; Bowman *et al.*, 1991b).

BEL1 *and* AG *interact to control integument development*

Previous studies of *bel1* mutants have suggested that *BEL1* is required for ovule integument morphogenesis (Modrusan *et al.*, 1994; Ray *et al.*, 1994; Reiser *et al.*, 1995; Robinson-Beers *et al.*, 1992). Our data confirm and extend these conclusions. We have provided additional proof for the hypothesis that the distinctive ovule morphology observed in *bel1* loss-of-function mutants is dependent on *AG* activity. A similar phenotype was observed in transgenic *Arabidopsis* lines where a *B. napus AG* gene was over-expressed (Ray *et al.*, 1994). Thus, excess *AG* activity appears to have a dominant negative effect on integument development. Taken together, these results suggest that the relative ratio of *BEL1* to *AG* activities is critical for proper development of the integuments. What could be the role of such BEL1–AG interactions in integument development? As has already been suggested, BEL1 may act as a negative regulator of *AG* function during integument development with *AG* serving no role at that stage of ovule development. However, even if this hypothesis is correct, the negative regulation of *AG* cannot be the only role of *BEL1* in integument development since integuments do not develop normally in most Bel1$^-$ ovules, even in the absence of *AG*. An alternative hypothesis is that *AG* has an active role in integument development but requires *BEL1* activity to function correctly at that stage.

Further roles for AG *and* BEL1 *during ovule development*

Ovule mutants lacking both sets of integuments, including *bel1* mutants, fail to develop normal embryo sacs (reviewed in Gasser *et al.*, 1998). We have shown that the embryo sac is still absent in ovules with normal integuments that lack *BEL1* (*ap2–6 ag-1 bel1–3* triple mutants; Figure 2). These results suggest that BEL1 may regulate a sporophytic function required for mega-gametogenesis. The exact role(s) of sporophytic tissue in embryo sac development is unclear, but investigation of the growing number of sporophytic female-sterile mutants having ovules with normal morphology but defective embryo sacs could yield many answers (Drews *et al.*, 1998; Schneitz *et al.*, 1997).

The decrease in the number of primordia initiated from the placental tissue of mutants homozygous for *ag* suggests a defect in the initiation of ovule primordia. A role for *AG* in the initiation of ovule primordia is supported by the presence of *AG* mRNA in the placenta before and during ovule initiation (Bowman *et al.*, 1991a; Reiser *et al.*, 1995). Alternatively, the reduction in the number of ovule primordia could be due to abnormal placental development in the absence of *AG*.

Experimental procedures

Plant materials and growth conditions

Lines of *Arabidopsis thaliana* used for the construction of double and triple mutants were: *ag-1* (CS25; Landsberg *erecta* [Ler] ecotype; gift from M. Koornneef, Wageningen Agricultural University, The Netherlands); *ap2–2* (CS3082; Ler; gift from E. Meyerowitz, California Institute of Technology, Pasadena, CA, USA); *ap2–6* (CS6240; Columbia-2 [Col-2] ecotype; Kunst *et al.*, 1989), *bel1–1* (CS3090; Ler; gift from C. Gasser, University of California, Davis, CA, USA); *bel1–3* (CS8545; Wassilewskija [WS]

ecotype); and *ap2–6 bel1–3* (Col-2 and WS; Modrusan *et al.*, 1994). Growth conditions were as in Modrusan *et al.* (1994).

Light and scanning electron microscopy

Whole-mount squashes of ovules were prepared as in Schneitz *et al.* (1995) and photographed under differential interference contrast optics using a Leitz DRB (Leica, Wetzlar, Germany) microscope with Kodak Ektachrome 160 ASA film (Eastman Kodak, Rochester, NY, USA).

SEM samples were prepared as in Modrusan *et al.* (1994). Outer whorl carpel-sepals were dissected from the flowers, mounted on stubs, coated with gold-palladium in a SEMPrep2 sputter coater (Nanotech, Manchester, UK) and observed using a Cambridge 250T scanning electron microscope (Leica, Wetzlar, Germany) with an accelerating voltage of 20 kV.

Construction and identification of double and triple mutants

ap2–6 bel1–3 *and* ap2–2 bel1–1 *double mutants.* *ap2–6 bel1–3* double mutants have been constructed previously (Modrusan *et al.*, 1994). To construct *ap2–2 bel1–1* double mutants, pollen from *bel1–1* flowers was used to fertilise *ap2–2* plants. *ap2 bel1* double mutants were identified in their respective F2 populations by screening $Ap2^-$ plants for $Bel1^-$ ovules. These results were confirmed through PCR and Southern hybridisation (see below).

ap2–6 ag-1 *and* ap2–2 ag-1 *double mutants.* Wild-type plants from an Ag^- segregating population were used as female parents in crosses to *ap2–6* mutants. $Ap2^-$ plants from the resulting F2 populations were used as female parents in crosses to *ap2–6 bel1–3* double mutant flowers to construct the triple mutant as described below. PCR was used to isolate *ap2–6 ag-1* double mutant from the triple-segregating population so that the *ap2–6 ag-1* double mutant and the *ap2–6 ag-1 bel1–3* triple mutant would have similar genetic backgrounds (see below). *ap2–2 ag-1* double mutants were constructed in the same manner but, since *ag-1*, *ap2–2* and *bel1–1* mutants are all of the Ler ecotype, they could be used directly for analysis.

ap2–6 ag-1 bel1–3 *and* ap2–2 ag-1 bel1–1 *triple mutants.* We anticipated that *ap2–6 ag-1 bel1–3* triple mutant flowers would be very similar in phenotype to *ap2–6 ag-1* double mutant flowers. Thus, the F2 progeny from the cross of *ap2–6 bel1–3* plants to *ap2–6/ap2–6 AG/ag-1* heterozygotes were grown and screened for plants with an $Ap2^-$ Ag^- phenotype. PCR and Southern hybridisation were used to identify triple mutants. *ap2–2 ag-1 bel1–1* triple mutant plants were identified amongst the F2 plants with an $Ap2^-$ Ag^- phenotype using PCR (see below).

DNA isolation, polymerase chain reaction and gel blot analysis

Crude DNA for PCR reactions was isolated from single *Arabidopsis* leaves (Edwards *et al.*, 1991). PCR was performed using Taq polymerase (Life Technologies, Burlington, ON, Canada) and a Perkin Elmer thermocycler (Perkin Elmer, Norwalk, CT, USA). Since the *bel1–3* allele resulted from insertion of a T-DNA carrying the *NPTII* gene (Modrusan *et al.*, 1994; Reiser *et al.*, 1995), the absence of *NPTII* was used to identify plants homozygous for *BEL1* in a population segregating *bel1–3. NPTII* was detected via PCR using right and left oligonucleotide *NPTII* primers (gift from R. Datla, Plant Biotechnology Institute, Saskatoon, SK, Canada). For the detection of the *bel1–3* allele, PCR was performed using oligonucleotide primers on either side of the approximately 10kb T-DNA insertion. The 5′ primer, 5BEL, started 195 bp downstream of the *BEL1* transcription start site (5′-ATGGCAGGGTCATGATCACC-3′), while the 3′ primer, R2, started at nucleotide –1055 (5′-TTGCATAGTCTCATGGCAG-3′). Amplification was performed with an annealing temperature of 54°C. *BEL1/BEL1* or *BEL1/bel1–3* plants gave a band of 860 bp, whilst *bel1–3/bel1–3* plants gave no product. A second 5′ primer, BELAT2, starting at nucleotide –750 (5′-AGACATGGCAAGAG-3′), was used with 5BEL under the same conditions as a control for the quality of the DNA preparations.

Plants that did not amplify the 5BEL-R2 fragment after several trials were selected as putative *bel1–3/bel1–3* homozygotes and were confirmed through Southern hybridisation. Single plant DNA preparations were isolated using the CTAB method of Dean *et al.* (1992) and digested with *Hin*dIII. The probe was derived from the wild-type BELAT2-R2 PCR fragment described above using random primer labelling (Life Technologies, Burlington, ON, Canada). The BELAT2-R2 fragment overlaps the 3′ end of a 2.9 kb *Hin*dIII fragment within *BEL1* and a 4–5 kb fragment in *bel1–3.*

Unlike *bel1–3, bel1–1* resulted from a point mutation (Reiser *et al.*, 1995) that created a new restriction site for *Taq*I (New England Biolabs, Beverly, MA, USA). A region surrounding the site of the point mutation was amplified using PCR and then incubated with *Taq*I. Those plants where the fragment was completely cleaved were identified as *bel1–1*. The PCR 5′ and 3′ primers used were located at –749 (AT3: 5′-GAGAGACATGGCAAGAGATCAG-3′) and –1229 (WT3′: 5′-GAGCATGGAGAGCAACTTGG-3′), respectively. PCR amplification was performed with an annealing temperature of 58°C.

Statistical analyses

Carpel-sepals were removed from several flowers of each plant and mounted together on stubs for SEM analyses. The average number of ovules per flower was calculated for each stub and treated as a random sample (*ap2–6* = 6 stubs, *ap2–6 bel1–3* = 16 stubs, *ap2–6 ag-1* = 21 stubs, *ap2–6 ag-1 bel1–3* = 16 stubs). The data set obtained for each of the four ovule types in the four mutants was subjected to a single factor analysis of variance (ANOVA) followed by Tukey pairwise comparisons using Systat (Wilkinson, 1988) to determine if the sample means were identical. Differences that are statistically significant have been noted in the text.

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Arabidopsis seed stock numbers: *ag-1* = CS25, *ap2–2* = CS3082, *ap2–6* = CS6240, *bel1–1* = CS3090, *bel1–3* = CS8545.