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 meagalgetogenesis (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
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 bel1–3α

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Number of flowers</th>
<th>Ovule type</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mature</td>
<td>Wild type</td>
<td>Flo 10</td>
<td>Bel1−</td>
<td>Immature</td>
</tr>
<tr>
<td>ap2–6β</td>
<td>172</td>
<td>94 (11 715)</td>
<td>1 (97)</td>
<td>0</td>
<td>0</td>
<td>&lt; 1 (31)</td>
</tr>
<tr>
<td>ap2–6 bel1–3c</td>
<td>23</td>
<td>0</td>
<td>0</td>
<td>70 (460)</td>
<td>26 (173)</td>
<td>0</td>
</tr>
</tbody>
</table>

*αNumber of ovules are in parentheses.
*βDissection microscope, includes both locules.
*γSEM data, one locule per flower.

Table 1b. Percentage of ovule types found on the carpel-sepals of mutantsαβ

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Number of flowers</th>
<th>Ovule type</th>
<th></th>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mature</td>
<td>Wild type</td>
<td>Flo 10</td>
<td>Bel1−</td>
<td>Immature</td>
</tr>
<tr>
<td>ap2–6</td>
<td>34</td>
<td>49 (650)</td>
<td>6 (81)</td>
<td>0</td>
<td>5 (66)</td>
<td>16 (209)</td>
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<tr>
<td>ap2–6 bel1–3</td>
<td>59</td>
<td>0</td>
<td>0</td>
<td>6 (95)</td>
<td>31 (470)</td>
<td>30 (449)</td>
</tr>
<tr>
<td>ap2–6 ag–1γ</td>
<td>63</td>
<td>33 (618)</td>
<td>3 (68)</td>
<td>0</td>
<td>6 (119)</td>
<td>54 (1021)</td>
</tr>
<tr>
<td>ap2–6 ag–1 bel1–3</td>
<td>70</td>
<td>1 (20)</td>
<td>0</td>
<td>0 (1)</td>
<td>11 (272)</td>
<td>76 (1832)</td>
</tr>
</tbody>
</table>

*αSEM data.
*βNumber of ovules are in parentheses.
*γIsolated from the triple-segregating population.

t 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). Carpels-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 megagametophyte. 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 bel1 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

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 (CS8846; Wassilewskija [WS]).

from an Ag– segregating population were used as female parents 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 mutants 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/bel1–1 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 thebel1–3 allele resulted from insertion of a T-DNA carrying the 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 at nucleotide –209 (5′-TTGCATAGTCTCATGGCAG-3′). Amplification was performed with an annealing temperature of 54°C. BEL1/bel1–1 or BEL1/bel1–3 plants gave a band of 860 bp, whilst bel1–1/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 bel1–1/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 HindIII. 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 HindIII 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 TaII (New England Biolabs, Beverly, MA, USA). A region surrounding the site of the point mutation was amplified using PCR and then incubated with TaII. Those plants where the fragment was completely cleaved were identified as bel1–1. The PCR 5′ and 3′ primers used were located at –479 (AT3: 5′-GAGAGACATGGGCAAGATCG-3′) and –1229 (WT3′: 5′-GAGAGACATGGGCAAGATCG-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 = 8 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.