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# Dissection of sexual organ ontogenesis: a genetic analysis of ovule development in *Arabidopsis thaliana*

**Kay Schneitz[†,*], Martin Hülskamp[‡], Steven D. Kopczak and Robert E. Pruitt**

Department of Molecular and Cellular Biology, The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA

[†]Present address: Institute of Plant Biology, University of Zürich, Zollikerstrasse 107, CH-8008 Zürich, Switzerland
[‡]Present address: Institut für Entwicklungsgenetik, Eberhard-Karls Universität Tübingen, Spemannstrasse 37-39, D-72076 Tübingen, Germany
*Author for correspondence (e-mail: schneitz@botinst.unizh.ch)

## SUMMARY

**Understanding organogenesis remains a major challenge in biology. Specification, initiation, pattern formation and cellular morphogenesis, have to be integrated to generate the final three-dimensional architecture of a multicellular organ. To tackle this problem we have chosen the ovules of the flowering plant *Arabidopsis thaliana* as a model system. In a first step towards a functional analysis of ovule development, we performed a large-scale genetic screen and isolated a number of sterile mutants with aberrant ovule development. We provide indirect genetic evidence for the existence of proximal-distal pattern formation in the *Arabidopsis* ovule primordium. The analysis of the mutants has identified genes that act at an intermediate regulatory level and control initiation of morphogenesis in response to proximal-distal patterning. A second group of genes functions at a subordinate control level and regulates general cellular processes of morphogenesis. A large group of male and female sterile mutants shows defects restricted to early or late gametogenesis. In addition, we propose that the mature ovule obtains its overall curved shape by at least three different processes that act in only one domain of the ovule.**

Key words: *Arabidopsis*, floral development, morphogenesis, organogenesis, ovules, pattern formation

## INTRODUCTION

The detailed mechanisms governing organogenesis, particularly in plants, remain largely elusive. Flowers, for example, contain several types of floral organs, and a great deal has been learned about how the identity of the floral organs is established (Coen and Meyerowitz, 1991; Ma, 1994; Weigel and Meyerowitz, 1994). However, it is not understood how the three-dimensional architecture of an organ is set up and maintained once it is committed to a particular fate.

To address this problem we turned to the ovule of *Arabidopsis thaliana* as a model system. A variety of properties make this simply structured yet highly differentiated organ particularly useful to work with and its development has been well documented (Mansfield et al., 1991; Modrusan et al., 1994; Robinson-Beers et al., 1992; Schneitz et al., 1995). The ovules are major female reproductive structures with 40 to 50 ovules located within the gynoecium. The *Arabidopsis* ovule is of the common *Polygonum*-type (Bouman, 1984; Reiser and Fischer, 1993; Willemse and van Went, 1984). Each ovule contains a seven-celled embryo sac at maturity. One of these cells is the egg cell proper, which, upon fertilization, generates the zygote and the plant embryo. Two integuments envelop the embryo sac. They will eventually form the seed coat or testa. A stalk-like structure, the funiculus, connects the ovule to the placental region of the gynoecium (Fig. 1). Due to the alternation of generations typical of the plant life cycle, the mature ovule is composed of tissues differing in the ploidy level of their cells. While the embryo sac is haploid, representing the (female) gametophytic phase of the life cycle, the rest of the ovule is diploid forming the sporophytic part of the organ.

One way to approach organogenesis at the functional level is to genetically dissect the process and to study systematically a large number of mutants. In this respect only little is known about ovule development. In particular, simultaneous cosuppression of two genes, *FBP7* and *FBP11*, results in a conversion of ovule into carpel tissue in petunia (Angenent et al., 1995; Colombo et al., 1995), while in tobacco two mutants have been reported with a similar phenotype (Evans and Malmberg, 1989). In *Arabidopsis*, genes like *APETALA2* (*AP2*) (Jofuku et al., 1994; Modrusan et al., 1994), *BELL1* (*BEL1*) (Modrusan et al., 1994; Ray et al., 1994; Reiser et al., 1995; Robinson-Beers et al., 1992), *AINTEGUMENTA* (*ANT*) (Elliott et al., 1996; Klucher et al., 1996) and *ABERRANT TESTA SHAPE* (*ATS*) (Léon-Kloosterziel et al., 1994) seem important for early functions while the floral homeotic gene *SUPERMAN* (*SUP*) (Bowman et al., 1992; Sakai et al., 1995) acts at later stages (Gaiser et al., 1995) as does *SHORT INTEGUMENTS* (*SIN1*) (Lang et al., 1994; Robinson-Beers et al., 1992).

In this study we have attempted a systematic analysis of ovule development. To identify genes important for ovule development we performed a large-scale single-line mutagen-

esis. We screened through nuclear-recessive sterile mutants to identify candidates showing defects in ovule ontogenesis. We have isolated many novel mutants as well as additional alleles of most of the previously identified genes. Based on a systematic analysis of the phenotypes we define several classes of mutants and identify distinct steps of the process of ovule organogenesis. The data allow several general conclusions concerning ovule development in *Arabidopsis*.

## MATERIALS AND METHODS

### Plant material and mutagenesis

Plants were either grown as described by Schneitz et al. (1995) or on autoclaved Florabella cactus soil (Klasmann-Deilmann, Geeste, Germany) beneath OSRAM 58W/72 Biolux fluorescent bulbs (OSRAM, Winterthur, Switzerland) in continuous light (about 100 $\mu E/m^2$/second at pot height) in a constant temperature room at 22°C. Seeds were mutagenized with ethyl methanesulphonate (EMS) essentially as described by Mayer et al. (1991). The wild-type strain used was *Arabidopsis thaliana* (L.) Heynh. var. Landsberg (*erecta* mutant). The $M_2$ families of approximately 15000 single $M_1$ lines were screened. We screened through nuclear-recessive sterile mutants to identify those showing defects in ovule ontogenesis. Families that segregated sterile plants were rescreened at least once. The type of sterility was checked with reciprocal outcrosses using the male-sterile line TH154 (Schneitz et al., 1995) and *Ler* wild-type plants. We investigated ovules of female-sterile (fs) as well as male and female-sterile (ms/fs) lines. Fs lines were sorted by phenotype. Allelism tests were performed with mutants showing a similar phenotype. Tests with *bel1* were done using a previously isolated allele (Robinson-Beers et al., 1992). Tests for allelism with *ant*, *ino*, *lug* and *sup* were performed with appropriate lines (Bowman et al., 1992; Elliott et al., 1996; Gaiser et al., 1995; Liu and Meyerowitz, 1995) and *hll*, *ant*, *bel* and *ino* alleles were also complementation tested against one another. Because of their similar phenotype the *sin1* (Lang et al., 1994; Robinson-Beers et al., 1992) and *lal* mutants were tested for complementation and the result showed that they are not allelic. The *HLL* and *SUB* genes were mapped to the upper region of chromosome 1 (K. Schneitz, unpublished data). Because *SIN1* maps to the top of chromosome 1 (Lang et al., 1994) cross-wise allelism tests have been performed showing that *SIN1*, *SUB* and *HLL* are different genes. The *bag*, *mog* and *ucn* mutants are classified on the basis of their distinct phenotypes. The ms/fs lines could be propagated through the heterozygous siblings of individual M2 families. No complementation tests were done with these lines. Ovule preparations were only performed with mutant lines which had been rescreened once or twice.

### Ovule preparations, microscopy and art work

Whole-mount ovule preparations and staging have been described by Schneitz et al. (1995). Preparations were examined using a Zeiss Axioplan microscope (Carl Zeiss, Oberkochen, Germany) with differential interference contrast (DIC) optics and pictures were taken on Kodak Ektachrome 160T slide film (Eastman Kodak, Rochester, NY). Slides were scanned by a local graphics bureau and put on compact discs (CD) in Kodak Photo-CD format.

For scanning electron microscopy (SEM), freshly opened flower buds were immersed in Jauch's fixative (70% acetone, 2% glutaraldehyde, in $H_2O$) and fixed for 6 to 12 hours at room temperature. The tissue was washed 10 times in 70% acetone, rehydrated through an acetone series in cacodylate buffer (50 mM sodium cacodylate pH 7.0), postfixed in 2% osmium tetroxide in cacodylate buffer for 2 hours at room temperature, washed twice in cacodylate buffer for 15 minutes and dehydrated through an acetone series. Subsequently, critical point drying was performed, the specimens were mounted on stubs and the ovules were dissected free. The tissue was sputtered with gold and examined with a Hitachi S4100 field emission scanning electron microscope (Nissei Sangyo Co., Ltd.). Pictures were taken on Kodak TMAX 100 Pro film. Negatives were scanned using a UMAX Power Look PS-2400X flatbed scanner (UMAX Data Systems, Inc.). Pictures were processed for publication on a Power Macintosh 8100/110 computer (Apple, Inc. Cupertino, CA) using Adobe Photoshop 3.0.5 (Adobe Systems, Inc. Mountain View, CA) and Macromedia Freehand 5.0.1 (Macromedia, Inc. San Francisco, CA) software. Printouts were generated on a FUJIX Pictrography 3000 color printer (Fujifilm Dielsdorf Ltd., Dielsdorf, Switzerland).

## RESULTS

### Wild-type ovule development in *Arabidopsis*

*Arabidopsis* ovule development has been well described (Mansfield et al., 1991; Modrusan et al., 1994; Robinson-Beers et al., 1992; Schneitz et al., 1995) and a staging system has been put forward (for details see Schneitz et al., 1995). Therefore we provide only a brief summary here (Fig. 1A-D). Visible morphological development begins with the formation of a small conical protuberance from the placenta (stages 1-I/II). During stage 2-I the megaspore mother cell appears subepidermally within the nucellus. Subsequently meiosis/cytokinesis leads to four haploid megaspores (stage 2-V) and an inner and an outer integument initiate from the chalazal region (stage 2-II/III). They are entirely of epidermal

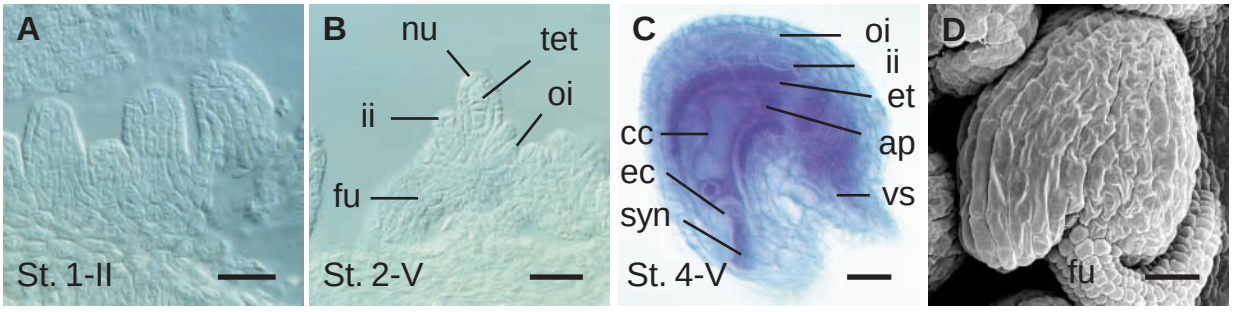


**Fig. 1.** Ovule development in *Arabidopsis thaliana*. (A-C) Mid-sagittal optical sections through whole-mount ovules. (A,B) Young ovules of Landsberg *erecta* wild-type plants. (A) Primordium at stage 1-II consisting of a homogeneous group of cells. (B) An ovule at stage 2-V. One can recognize the nucellus (nu) with a tetrad (tet), the inner (ii) and outer (oi) integuments and the funiculus (fu). (C) Mature ovule of the male-sterile line TH154 (Hülskamp et al., 1995) at stage 4-V (about 1-2 days after anthesis). Note the typical kinked (amphitropous) shape of mature ovules. At this time embryo and endosperm development is well underway in wild type (Schneitz et al., 1995). The embryo sac consists of two synergids (syn), the egg cell (ec), the large central cell (cc) and three antipodal cells (ap). The inner integument differentiates the endothelium (et). The vascular strand (vs) has developed. (D) A scanning electron micrograph of an ovule of about the same stage as the one in C. Scale bars, 20 μm.

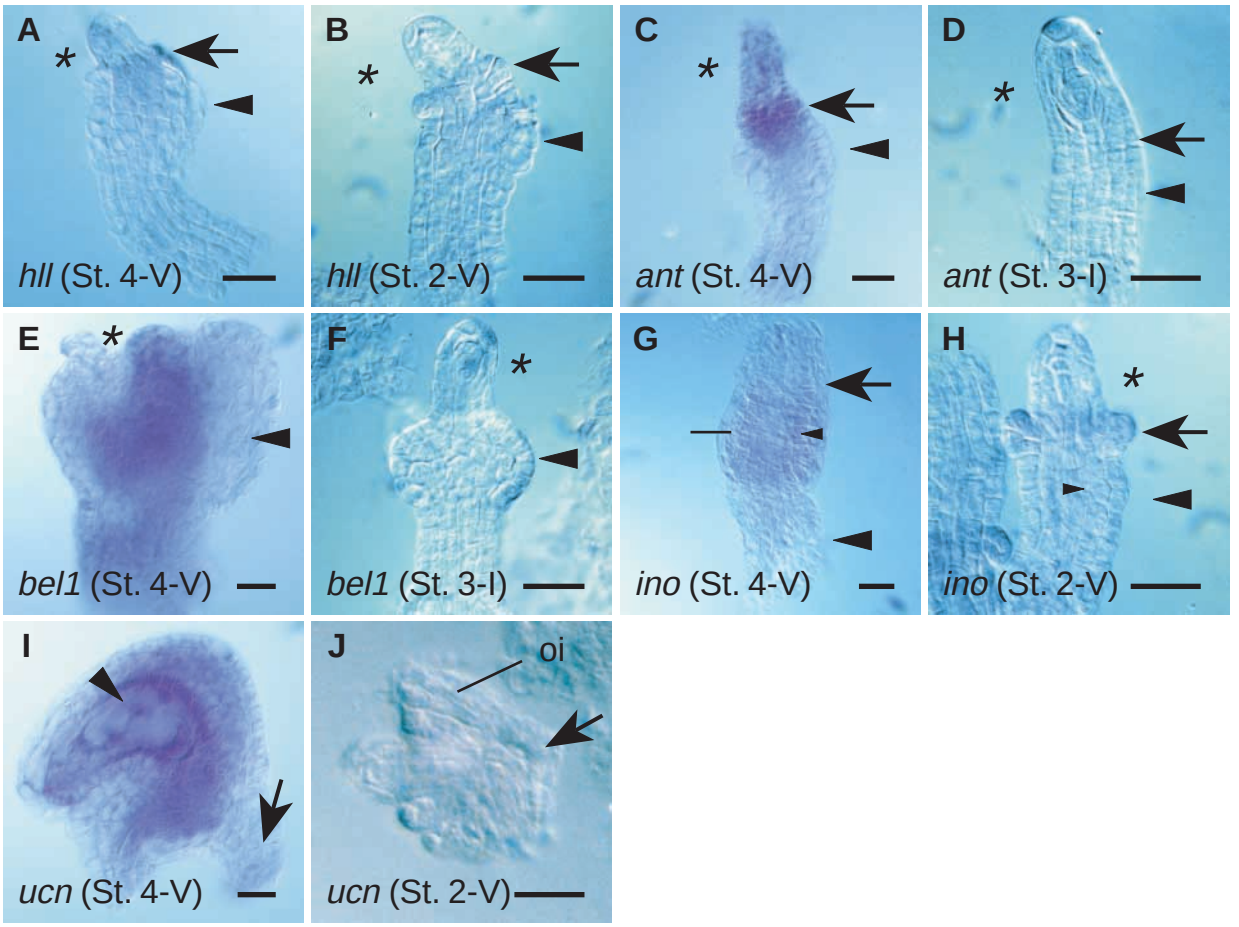


**Fig. 2.** Mutant phenotypes of the early-acting members of the *SMD* class as seen in optical sections of whole-mount ovule preparations. Mature (A,C,E,G,I) and young (B,D,F,H,J) ovules from each mutant are depicted with the specific stages indicated. The asterisks highlight the nucellar region, the large arrows and arrowheads indicate the inner and the outer integument domains, respectively. (A,B) *hll*; allele 79F2. (B) Within the nucellus the tetrad is at a different plane of focus. (C,D) *ant*; allele 72F5. (C) Note the lack of inner integument morphogenesis and the enlarged epidermal cells at the outer integument position. (D) There is a mono-nuclear embryo sac within the nucellus and there is no sign of integument development yet. (E,F) *bel1*; allele 1460. (E) Instead of an outer integument large bulges have developed. (F) A mono-nuclear embryo sac is apparent. Initiation of the bulges comprises subepidermal tissue. (G,H) *ino*; allele 46E4. (G) The small arrowhead denotes the endothelium and the line the third inner integument cell layer. (H) The nucellus carries a tetrad which is out of focus. The small arrowhead indicates the correct cell divisions occurring subepidermally within the outer integument domain. (I,J) *ucn*; mutant 135D12. (I) The arrow marks the protrusion and the arrowhead indicates endosperm development. (J) The extra-structure is indicated by the arrow. Scale bars, 20 μm.

origin and will form two sheets, each primarily consisting of two cell layers, that grow around the nucellus. Stages 3-I to 3-VI relate to the period of embryo sac formation in the nucellus and the maturation of the integuments. Only the proximal-most megaspore survives and undergoes further development to form the seven-celled embryo sac thereby replacing most of the nucellar tissue. Asymmetric growth of the integuments contributes to the characteristic curvature of the ovule. By the end of stage 3 the vascular strand has appeared in the funiculus. Stages 4-I to 4-V cover fertilization and the initial steps of endosperm development and embryogenesis. By this time the final amphitropous shape of the ovule is achieved.

## Identification of mutants defective in ovule development

Here we report on the initial characterization of 458 independent mutant lines. On a morphological basis one can discriminate three major phenotypic classes (Table 1). The sporophytic and megagametogenesis-defective (*smd*) class consisting of 28 mutants which represent 11 different loci (see Materials and methods). Mutants of this class usually show defects in the diploid and haploid tissues of the ovule. The megasporogenesis-defective (*msd*) group encompassing 270 mutants with defects apparently confined to spore development. The embryo sac-defective (*emd*) class consists of 160 mutants which show defects during gametophyte development. Mutants belonging to the *smd* class are female sterile (usually bearing relatively normal pollen) with one exception (see below). Mutants belonging to the *msd* and *emd* classes, however, produce either no pollen, a greatly reduced amount of pollen, or degenerate pollen and are therefore male and female sterile. In the following description of the different mutant phenotypes we focus on the defects detected within the ovule.

## Phenotypes of the *smd* class mutants

Mutations in five ‘early-acting’ genes either result in the

**Table 1. Phenotypic classes**

| Class | No. of mutants | No. of genes | Type of sterility |
|---|---|---|---|
| SMD | 28 | 11 | fs† |
| MSD | 270 | ND* | ms/fs |
| EMD | 160 | ND* | ms/fs |

*Not determined.
†With one exception being ms/fs.

absence of, or, in one case, in supernumerary ovule structures. The defects usually become visible by mid-stage 2. Mutations in six 'late-acting' loci, however, lead to an altered morphogenesis of the ovule and the alterations generally appear during early-to-mid-stage 3.

### *HUELLENLOS* (*HLL*)

We isolated two alleles of *HLL*. The phenotype of the stronger allele 79F2 is depicted in Fig. 2A,B. Apparent defects are restricted to the ovules. At maturity *hll* ovules are small and lack an embryo sac, both integuments and a mature vascular strand. The defects first become visible around stage 2-II/III. The inner integument does not form beyond the first epidermal cell division. Occasionally even this first cell divsion is absent. With respect to outer integument development, cell enlargement often occurs but epidermal cell divisions are usually not detected. Gametogenesis seemingly stops after tetrad formation. Therefore it looks like the development of the ovule ceases at or shortly after stage 2-V. The tissue may start to degenerate, the nucellar region usually being affected first.

### *AINTEGUMENTA* (*ANT*)

Several mutations in *ANT* have recently been described (Elliott et al., 1996; Klucher et al., 1996). We isolated four new alleles and the phenotype of a strong allele (72F5) is shown in Fig. 2C,D. Until stage 3-I development within the nucellus proceeds normally (mononuclear embryo sac), however, subsequent development ceases in this element. Within the central region in many cases there is no sign of integument initiation by this stage. Occasionally some development in the proximal half of the domain is apparent. The subepidermal cell divisions have occurred. In addition, there is some enlargement of epidermal cells indicating that outer integument development occurs to a certain extent (data not shown). At stage 4-V practically all ovules show enlarged epidermal cells at the outer integumentary position. This suggests that some outer integument development occurs quite regularly albeit somewhat delayed. However, there is still no morphological sign of inner integument development. The nucellus appears elongated (compare Fig. 2C with 2A) and lacks an embryo sac. Within the funiculus no mature vascular strand is observed.

### *BELL* (*BEL1*)

Three *bel1* alleles of similar strength were isolated and the phenotype of one (*1460*) is summarized in Fig. 2E,F. Its description does not deviate significantly from reports on other *bel1* alleles (Modrusan et al., 1994; Ray et al., 1994; Robinson-Beers et al., 1992). Early ovule development in this mutant is normal. Development within the nucellus starts to deviate after stage 3-I (similar to the situation in *ant-72F5*) when embryo sac ontogenesis ceases. Much earlier (by stage 2-II) a defect is seen in the central domain. Instead of integument initiation, large bulges appear at the position where normally the outer integument is initiated. These bulges consist of epidermal and subepidermal cells and they continue to grow until they extend just above the nucellus. No inner integument development is observed. The stage 4-V ovules show an orthotropous stature (erect) rather than the amphitropous pattern and a broader than normal funiculus. No embryo sac is observed in the nucellus. At very late stages the bulges may develop into carpelloid structures (not shown).

### *INNER NO OUTER* (*INO*)

A brief description of an *ino* mutant has appeared in the literature (Gaiser et al., 1995). Figure 2G,H shows the phenotype of a strong allele (46E4), one of seven new alleles that were isolated. As in the previous examples, early development appears normal and there is a difference in timing of the appearance of defects in the nucellus and the integuments. While at stage 4-V the embryo sac is lacking, its development never stops before the formation of a mono-nuclear embryo sac and a four-nuclear embryo sac is often discernible. Morphogenesis of the central region is characterized by the lack of the outer integument. While the epidermal cell enlargement typical of early outer integument development occurs, no oblique or periclinal cell division is apparent and development does not proceed further. However, the subepidermal cell proliferation in this region does take place indicating that these two processes are separable. While at maturity the *ino* ovules exhibit a bending, they do not show the typical amphitropous shape (see Discussion). Except for its shape, the inner integument develops normally. At stage 4-V the endothelium is visible and the third layer, originating from the endothelium at stage 3-VI (Schneitz et al., 1995), exists.

### *UNICORN* (*UCN*)

The single *ucn* mutant we have isolated (135D12) features an astonishing phenotype (Fig. 2I,J). At stage 4-V a horn-like excrescence from the outer integument is present at a distinct proximal-distal location. This bump may vary in size from a mere swelling of a few epidermal cells (2-4 cells in an optical cross section) to a considerable protrusion consisting of two or three cell layers. Often however the layering is not clear cut. The embryo sac is usually missing but occasionally is normal. The mutant is semi-sterile and seeds can be obtained. This extra excrescence appears after stage 2-III when both integuments are initiated and embryo sac morphogenesis may fail at various times after stage 3-I.

### *STRUBBELIG* (*SUB*)

We have isolated four mutant alleles of *SUB* and they all display similar phenotypes. Fig. 3A-C shows aspects of the phenotype of the allele 88C12. At maturity *sub* ovules exhibit several aberrant features. Most prominently the outer integument often forms several protrusions at the distal tip. In some more extreme cases the outer integument does not encapsulate the inner integument and the nucellus but rather generates an open-folded sheet-like structure (Fig. 3C). Inner integument morphogenesis is relatively normal, developing characteristics like the endothelium, for example. The funiculus, however, is affected because very often no mature vascular strand is discernible. The embryo sac is variably influenced. Its develop-

ment usually proceeds beyond the mono-nuclear embryo sac and often arrests only at the eight-nuclear stage before cellularization takes place. Some ovules look relatively normal and are sometimes fertile. Development of *sub* ovules starts to deviate from wild type only after stage 3-I or stage 3-II when the outer integument may not close at the distal (micropylar) tip and embryo sac development can become aberrant. In addition to the defects in the ovule and a slightly reduced amount of pollen *sub* plants exhibit wrinkled and twisted pistils and the plants look much shorter and stubbier than wild-type plants.

### *BLASIG* (*BAG*)

One *bag* mutant was identified (94G3; Fig. 3D,E,F). Stage 4-V ovules from *bag* mutants exhibit dramatic alterations. Except for the funiculus with the vascular strand no clear morphological features are recognizable in whole-mount preparations. Interestingly the ovules stick together tightly, a property that was never observed in wild type. At higher magnifications, cells are larger and roundish particular at the edges of the tissue. This can also be seen in scanning electron micrographs (Fig. 3E). An embryo sac is usually not visible. Young ovules (until about stage 2-III) appear normal except that the epidermal cells may look somewhat more roundish. Nevertheless, integument initiation and megaspore mother cells can be clearly observed. Shortly after stage 3-I one fails to detect clear morphological structures. Even though the ovules have already stuck to each other they continue to grow in size. Plants defective for *BAG* function are also smaller than wild-type, bear flowers that open prematurely and have sepals and petals of irregular shape that may appear glassy. The anthers have a somewhat reduced amount of pollen.

### *MOLLIG* (*MOG*)

We identified one mutant in the *MOG* gene (179A10; Fig. 3G,H). While the overall shape of an ovule is maintained, stage 4-V *mog* ovules display large, balloon-shaped cells in both the inner and the outer integuments and the cell layers are often not regular in appearance. This may lead to an aberrant layer structure in the integuments. The effects on embryo sac development vary from an unrecognizable patch of tissue to an occasional eight-nuclear embryo sac. Similarly, the mature vascular strand in the funiculus may or may not be present. The *mog* ovules appear normal until about stage 3-II when the first weak signs of larger integument cells become detectable. Similar to *sub* and *bag* mutants, *mog* plants show a pleiotropic phenotype. They have shorter internodes and therefore exhibit a more 'crowded' inflorescence. The pistils are thicker than normal and the stigmas bear a reduced number of papillar cells of smaller size. The stamens have short filaments and the anthers bear no pollen.

### *LAELLI* (*LAL*)

Mature ovules of the *lal* mutant resemble to some degree ovules of plants defective for *SIN1* (Lang et al., 1994; Robinson-Beers et al., 1992). However, as opposed to the *sin1* mutant the defects in *lal* plants are restricted to the ovule and the two mutants are not allelic (data not shown). We have isolated one *lal* mutant (196F6; Fig. 3I,J). Stage 4-V ovules of *lal* plants lack an embryo sac and show a much reduced outer integument with the nucellus and the inner integument protruding. The inner integument appears wild type. Early ovule development in the *lal* mutant appears normal until early stage 3-I. Within the nucellus embryo sac development proceeds until the four-nuclear stage. Older embryo sacs were not observed. By stage 3-III *lal* mutant plants bear ovules which show the mutant features. The phenotype suggests that either cell expansion or the anticlinal cell divisions within the outer integument is affected.

### *LEUNIG* (*LUG*)

The *LUG* gene was shown to exert a cadastral role during early flower development (Liu and Meyerowitz, 1995). Here we show that *LUG* has a previously unreported function during ovule development. We have isolated two new *lug* alleles and the phenotype of one (44D2) is presented in Fig. 3K,L. The phenotype of mature ovules defective in *LUG* function varies considerably. They often lack an embryo sac and it appears as if development simply stopped prematurely. Occasionally they show an enlarged nucellus and inner integument that extend distally beyond the tip of the outer integument. The defect first becomes evident after stage 3-I. The embryo sac rarely develops beyond the mono-nuclear stage and at stage 3-III the outer integument often remains open distally.

### *SUPERMAN* (*SUP*)

The floral homeotic gene *SUP* (Bowman et al., 1992; Sakai et al., 1995) was recently shown to have a function during ovule development (Gaiser et al., 1995). We have identified two new *sup* alleles and the analysis of the ovule phenotype leads to similar conclusions. Ovules of allele 198G3 are depicted in Fig. 3M,N. Most of the stage 4-V *sup* ovules show a relatively well developed embryo sac. However, instead of showing the amphitropous shape of wild-type ovules they appear straight from about midway through the proximal-distal extension of the integuments. This is due to excess growth of the outer integument in the anterior (adaxial) domain while the inner integument appears comparatively unaffected. At a more proximal position within the chalaza curvature of the ovule is observed and the funiculus also exhibits its typical S-shape. The defect becomes evident by stage 3-II when the outer integument elongates and demonstrates abnormal straight growth.

## The *msd* and *emd* classes

These two groups are characterized by mutants exhibiting defects restricted to gametogenesis. In all these mutants we cannot detect any obvious defects in the sporophytic tissue of the ovule.

We have isolated 270 mutants which fall into the *msd* class. At maturity they all show a similar phenotype of displaying no sign of embryo sac development. As an exemplary case we describe the mutant 170F5 (Fig. 4A,B) in more detail. At stage 4-V, either nothing or only a patch of tissue is visible instead of an embryo sac. This patch probably represents a degenerated tetrad because the tetrad usually forms in this mutant although subsequent embryo sac development fails. In 170F5, early megaspore development appears normal and meiosis proceeds until the formation of the tetrad. Embryo sac development is commonly absent. In only two out of over 70 ovules examined did we observe a two nuclear embryo sac with a vacuole. We therefore conclude that the defect in 170F5 occurs

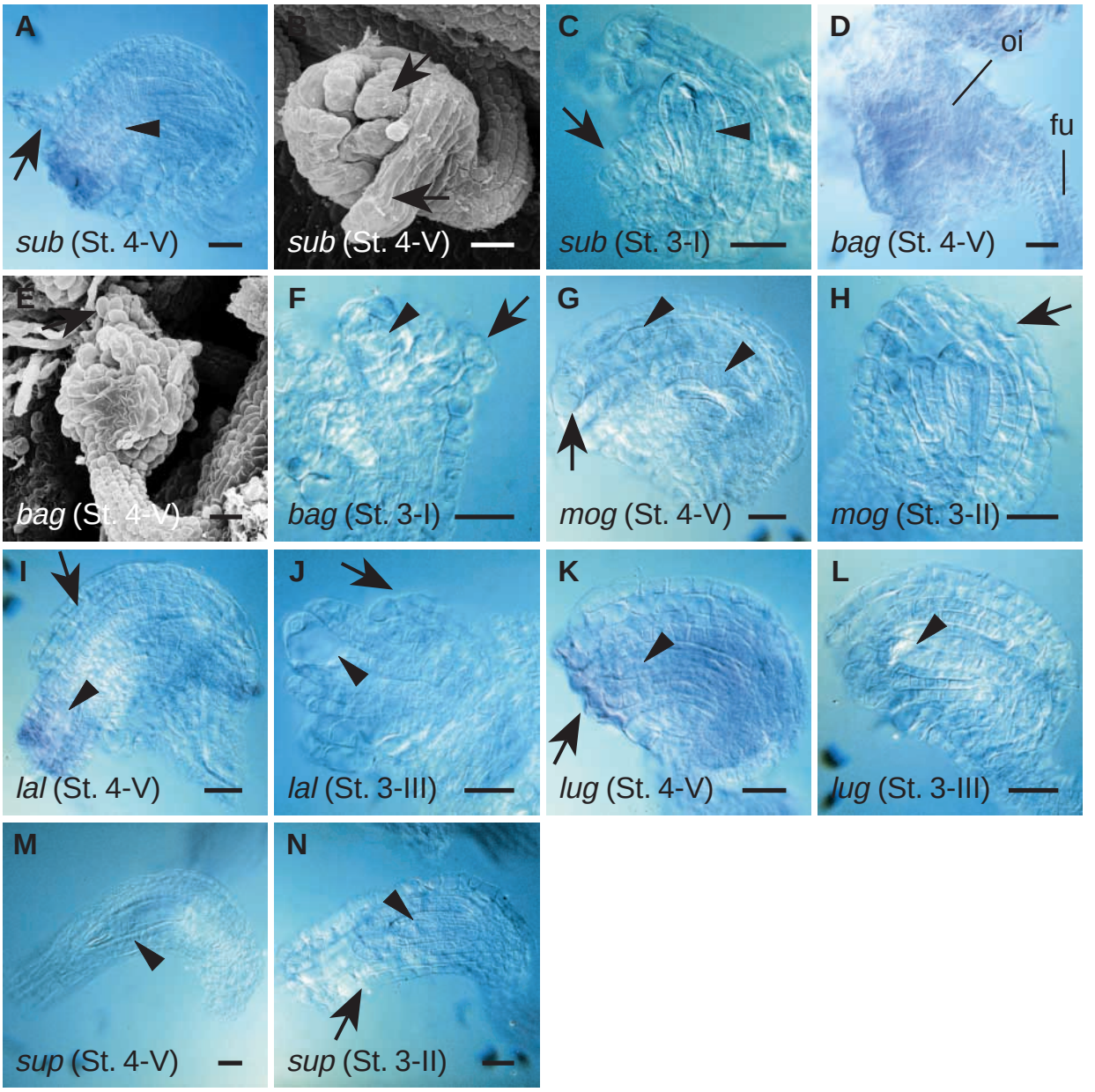


**Fig. 3.** Mutant phenotypes of the late-acting members of the *SMD* class. Optical sections through whole-mount ovule preparations are shown except for B and E which are scanning electron micrographs. The corresponding stages are indicated. (A-C) *sub*; allele 88C12. (A,B) Arrows highlight the distal integumentary protuberances. (A) Small arrowhead indicates lack of embryo sac. (C) The anterior side of the outer integument is strongly reduced (arrow). A mono-nuclear embryo sac is visible (arrowhead). (D-F) *bag*; mutant 94G3. (D) The funiculus and the main part of the outer integument are marked. (E) Note the roundish-shaped cells of the distal half of the integuments (for example, arrow). (F) The mono-nuclear embryo sac is slightly out of the focal plane (arrowhead) and the cells are already roundish (arrow). (G,H) *mog*; mutant 179A10. (G) Aberrant cell layers are apparent (arrowheads) and the cells may be larger or misformed (arrow). (H) Cell shape changes become visible (arrow). (I,J) *lal*; mutant 196F6. (I) The arrow marks the reduction of the outer integument. The embryo sac appears absent (arrowhead). (J) The arrowhead indicates the two-nuclear embryo sac. The outer integument is already shorter (arrow). (K,L) *lug*; allele 44D2. (K) The integuments are reduced (arrow) and no embryo sac is visible (arrowhead). (L) No embryo sac apparent (arrowhead). (M,N) *sup*; allele 198G3. (M) The integuments form a tube and the embryo sac seems normal (arrowhead). (N) A two nuclear embryo sac is visible (arrowhead). The asymmetric growth of the outer integument is already apparent (arrow). Scale bars, 20 μm.

prior to embryo sac formation some time during megaspore formation.

As in the *msd* class, the defects of the 160 mutants of the *emd* group seem restricted to gametogenesis. However, the defects become apparent during embryo sac development. Characteristically one observes a broad spectrum of defects even within a given mutant line, often ranging from an *msd*-type defect to a normal-looking embryo sac. Here we show two examples with a relatively narrow range of phenotypes. Fig. 4C depicts a stage 4-V ovule of the mutant 211E6. In nearly

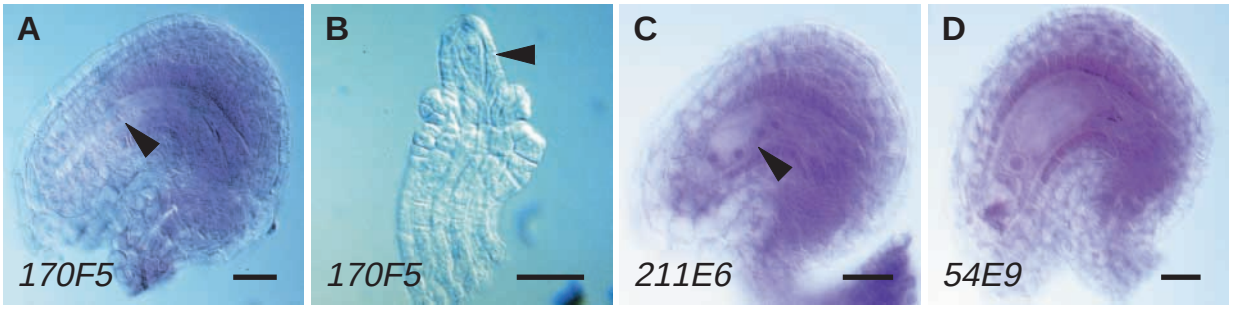


**Fig. 4.** The *MSD* and *EMD* classes. Optical sections through whole-mount preparations are depicted. The designation of the mutants is given. Mutant ovules of stage 4-V are shown except in B which shows a stage 2-IV ovule. (A,B) An *msd*-class example, 170F5. (A) The arrowhead indicates the absence of an embryo sac. (B) Note the occurrence of meiosis (arrowhead). (C) The *emd*-class example, 211E6. Embryo sac development arrested at the four-nuclear stage (arrowhead). (D) The *emd*-type mutant 54E9 with apparently normal embryo sac. Scale bars, 20 μm.

all 211E6 ovules, embryo sac development seems to be arrested at the four-nuclear (stage 3-IV) stage with an irregular spatial arrangement of the nuclei. Some ovules show a mature embryo sac. Fig. 4D shows a mature ovule of the mutant 54E9. Generally the embryo sac in this mutant appears normal but shows no sign that fertilization occurred. A few examples exhibit a large scrap of unidentifiable tissue instead of an embryo sac, which suggests that sometimes the embryo sac degenerates.

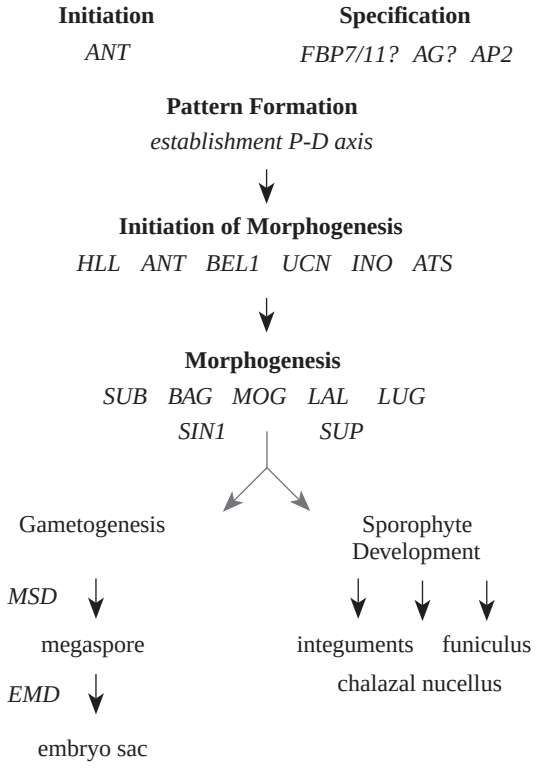


**Fig. 5.** Genetic model for ovule development. A flowchart highlights the different processes and the genes thought to be involved. The role of the genes involved in specification is inferred from the work of others (Angenent et al., 1995; Colombo et al., 1995; Modrusan et al., 1994; Ray et al., 1994; Reiser et al., 1995). *FBP7/11*? refers to hypothetical *Arabidopsis* orthologues. *ATS* has been described by others (Léon-Kloosterziel et al., 1994).

## DISCUSSION

Ovule development in *Arabidopsis* can be formally divided into several distinct processes: initiation, specification, pattern formation and morphogenesis (Schneitz et al., 1995). As a first step towards the identification of key factors involved in these processes we isolated 458 sterile mutants which show a defect during ovule development. The morphological analysis of the mutants suggests a hierarchy of genes responsible for initiating and directing morphogenesis in response to specification and pattern formation (Fig. 5).

### Specification and proximal-distal pattern formation in the *Arabidopsis* ovule

Even though a large mutant screen was performed several types of mutants are conspicuously absent from this collection. We did not isolate mutants with a defect in specification of ovule identity or pattern formation. We can only speculate about the reasons but one likely cause is genetic redundancy. For example, in petunia two MADS-box genes, *FBP7* and *FBP11* were identified that specify ovule identity (Angenent et al., 1995; Colombo et al., 1995). Cosuppression experiments with transgenic plants yielded conversions from ovules to carpelloid tissue. However, in these plants expression of both genes was absent. There are many MADS-box genes that are expressed in the young *Arabidopsis* ovule (Ma et al., 1991; Rounsley et al., 1995; Savidge et al., 1995) which may have such redundant functions. In addition, the analysis of a particular mutant allele of *AP2* implicated a function for this gene in ovule specification (Modrusan et al., 1994).

Earlier we put forward a hypothesis in which pattern formation in the ovule primordium results in an arrangement of three main pattern elements along a proximal-distal (P-D) axis (Schneitz et al., 1995). The distal element (D) gives rise to the nucellus, the central element (C) originates the chalaza, and the proximal element (P) forms the funiculus. Morphogenesis then produces element-specific differentiation. While not affecting the proposed proximal-distal patterning process per se (see below), some early-acting *SMD*-class mutants provide supporting evidence for the existance of such a process. Defects can be region-specific indicating that distinct regions (i.e., pattern elements) are set up and continue to develop independently from each other to a large extent. For

example, in *bel1* and *ino* mutants a central (chalazal) domain is severely affected very early on, but development within distal and proximal domains (nucellus and funiculus) proceeds relatively normally. Gametogenesis in the nucellus continues until after early embryo sac formation in both mutants, while funiculus development is usually quite normal. Similar arguments can be made with respect to *hll* and *ant*. Additional support stems from the investigation of the *BEL1* mRNA expression pattern (Reiser et al., 1995). At early stage 2, preceding the initiation of the integuments, *BEL1* transcripts accumulate in the central region of the ovule providing molecular evidence for a central pattern element.

While nucellar development proceeds largely independently of the other elements, the data also indicate that at very late stages (after stage 3-I) its independent mode of development is lost. This implies the importance of other ovular tissue, most likely the integuments, for embryo sac ontogenesis (see also Reiser and Fischer, 1993). The expression patterns of both *BEL1* and *ANT* support this notion. In both instances, the transcripts are not detected in the megaspore mother cell or the developing embryo sac (Elliott et al., 1996; Klucher et al., 1996; Reiser et al., 1995). However, integument and funiculus development is independent of the presence of an embryo sac as indicated by the *msd*-type mutants.

### The early-acting genes of the *smd* class are involved in region-specific initiation of morphogenesis along the P-D axis

We have identified mutations in five loci that seem to act at an intermediate level in the sequence of events outlined in Fig. 5. They probably function in response to pattern formation because in all mutants the defects become detectable during ovule mid-stage 2 after the P-D regionalization has become apparent. The earliest steps in the formation of a tissue, particularly the central-region-derived integuments, are impaired. While the typical initial cell shape changes occur, subsequent cell divisions do not, or only irregularly, take place and development ceases. This leads to the absence of entire structures. The mutants seem to be defective in what we conveniently call initiation of morphogenesis, as opposed to defects in cellular aspects of morphogenesis. The latter type of defect leads to a structure being present but having an altered appearance.

Mutations in *HLL* and *ANT* lead to a similar but not identical phenotype in the ovule. A major difference between the two mutants lies in the fact that plants defective for *ANT* show additional defects in other floral organs and bear a reduced number of floral organs, including the ovules (Elliott et al., 1996; Klucher et al., 1996). This suggests that *ANT* is repeatedly required during floral development and that it plays a role in floral organ and tissue initiation in general. This hypothesis is supported by the *ANT* mRNA expression pattern and the fact that the gene encodes a putative transcription factor with homology to the floral homeotic gene *AP2* (Elliott et al., 1996; Klucher et al., 1996; Weigel, 1995). The apparent defects of mutations in *HLL* are restricted to the ovule. No significant reduction of the number of floral organs has been observed in *hll* mutants (K. Schneitz, unpublished data). Experiments are currently underway that address the relationship between *HLL* and *ANT*.

The phenotypes of ovules from *bel1* and *ino* mutants differ from the previous two cases, particularly in the central and proximal regions. In *bel1* mutants the entire central region is affected. The inner integument seemingly fails to initiate and instead of an outer integument a structure arises which was previously characterized as integument-like (Modrusan et al., 1994; Robinson-Beers et al., 1992). As judged by the mode of initiation (epidermal and subepidermal tissues contribute to the bulges), the difference in cell characteristics (lack of large trapezoidal epidermal cells with prominent vacuoles at the outside), and the subsequent growth pattern, this structure shows no similarity to an outer integument and its identity is not known. The *BEL1* gene encodes a homeodomain-containing putative transcription factor (Reiser et al., 1995). Such a molecular function is compatible with the idea of *BEL1* being a regulatory gene which initiates morphogenesis in the central domain. The *ino* mutant specifically lacks an outer integument, while the inner integument seems to undergo normal development until stage 4-V. Both mutants indicate that development of the inner and outer integument is differentially controlled, corroborating ideas about a separate evolutionary origin of the two integuments (Stebbins, 1974; Stewart and Rothwell, 1993).

The fertile seed-shape mutant *aberrant testa shape* (*ats*) also falls into this early-acting class (Léon-Kloosterziel et al., 1994). At the time of integument initiation the two integuments are not distinguishable as separate structures. The boundary between the inner and outer integument is missing, resulting in a single 'fused' integument.

The defect in *ucn* leads to a supernumerary structure. In this mutant a protrusion forms at a specific location on the circumference of the ovule reminiscent of the initiation of the integuments (Robinson-Beers et al., 1992). The protrusion is generated very early in development, proximal to the outer integument and is often formed by enlarged epidermal cells. It is therefore a possibility that this protrusion represents a small ectopic integument which failed to undergo further development. If this interpretation is correct then *UCN* is a suppressor of integument initiation.

### The late-acting genes of the *smd* class affect cellular aspects of morphogenesis

Following patterning and initiation of morphogenesis the next hierarchical step involves the direction of morphogenesis proper. We identified six different mutants that show impaired morphogenesis. Typically the mutant phenotypes become apparent during ovule stage 3 and occasionally by the end of stage 2. Therefore, they probably act after morphogenesis has been initiated by the genes discussed in the previous section. It also indicates that the morphology of the cells of the ovule primordium is controlled by a different set of genes because the primordia appear normal in the mutants. The late-acting *smd* class genes appear to control general growth functions, in particular the regulation of cell shape and/or cell proliferation. In the case of the *bag* mutant the ovules stick together tightly, indicating that cell surface properties are also altered. With one exception (*lal*) the mutant defects are not restricted to the ovule. They either affect the entire plant like *sub*, *bag* or *mog* mutants or early flower development, like *lug* and *sup* (Bowman et al., 1992; Liu and Meyerowitz, 1995; Sakai et al., 1995). In the latter two cases the defects have also been interpreted as disruptions in growth processes (Gaiser et al., 1995; Liu and Meyerowitz, 1995; Sakai et al., 1995). However, the defects in the ovules of *sup* mutants are much more specific.

Mutations with such pleiotropic effects as observed in the former three mutants may be expected in genes that have a function in cellular differentiation. The observed effects on gametogenesis usually become visible after embryo sac ontogenesis is initiated. Either these effects are of secondary nature due to, for example, abnormal integument development or a given gene may also have a specific function during embryo sac development.

There is one more known mutant that belongs in this class. A mutation in *SHORT INTEGUMENTS* (*SIN1*), previously identified in our lab, causes a missing embryo sac and shorter integuments in a Landsberg *erecta* background (Robinson-Beers et al., 1992). The cell number is about normal but cell enlargement seems affected. However, when the *sin1* mutation is introduced into a Columbia background the inner integument is extroverted and often expanded (Lang et al., 1994). We have isolated two more putative *sin1* alleles but both were unfortunately lost.

### The mutants of the *msd* and *emd* classes show defects restricted to gametogenesis

A large and distinct gametogenesis-defective class consists of two groups, *msd* and *emd*, specifically affecting megaspore/embryo sac development within the nucellus. The lesions in the *msd* class most likely occur prior to the start of embryo sac development. Development in *msd*-type mutants fails to proceed beyond the megaspore or mono-nuclear embryo sac stage. Hence they seem to affect sporogenesis. The range of defects observed in *emd*-class mutants, often even within a mutant line, is not unknown in mutants affecting the embryo sac (Benavante et al., 1989; Huang and Sheridan, 1996; Kennell and Horner, 1985; Kermicle, 1971; Lin, 1978). Currently however, it does not allow for an easy interpretation of their function. The commonality resides in the defects becoming visible during embryo sac development. They may show aberrant numbers of nuclei, generally distorted morphology or pass through development until maturity but fail to proceed further (embryo/endosperm development). Mutants of both classes may show no, few or abnormal pollen and hence are male and female sterile. This indicates that they also function during pollen development. Therefore they open up roads to the study of sporogenesis and gametogenesis in general. The nuclear-recessive nature of the *emd* class suggests that many of these mutations may simply be leaky *msd*-type lesions. However, some of them could be involved in embryo sac development indicating that megagametophyte development depends to some extent on the sporophytic genome.

### The shape of the *Arabidopsis* ovule is achieved through multiple processes

A major aspect of ovule morphogenesis is the formation of the curved shape of the *Arabidopsis* ovule which is exemplified by the location of the distal tip (micropyle) next to the funiculus. This feature is shared by the majority of Angiosperm species (Bouman, 1984). Erect ovules are called orthotropous and curved ovules anatropous. Amphitropy refers to the curved nucellus and embryo sac that may appear on top of anatropy (Gifford and Foster, 1989).

Analysis of the *sup* mutant indicated that the anterior (adaxial) side of the outer integument is responsible for causing amphitropy (Gaiser et al., 1995) suggesting that a single gene, *SUP*, is largely responsible for the conversion of orthotropy to amphitropy. However, the *sup* mutant still exhibits an approximately right angle bend (Fig. 3M). This is probably due to the cell proliferation that still occurs in the posterior (abaxial) region of the chalaza beneath the outer integument. This differential cell proliferation is maintained in *ino* mutants (Fig. 2H) and they bear ovules with a bend similar to that seen in ovules defective in *SUP* function. Ovules of *bel1* mutants, lacking this differential cell proliferation in the chalaza, are orthotropous. Taken together the observations imply that at least three processes contribute to the curvature of the ovule, and that only the proximal half of the chalaza and the outer integument are involved. First, extra subepidermal cell divisions in the posterior half of the chalaza cause an initial bending of the ovule (hemitropy). Secondly, differential growth in the outer integument leads to anatropy. Thirdly, enlargement of cells from the anterior side of the base of the outer integument leads to the kink in the nucellus and embryo sac resulting in the amphitropous shape. The amphitropy of mature ovules from *msd*-class mutants support the notion that the embryo sac is not actively involved in this process. In addition to the ovule-autonomous processes described above for *Arabidopsis* it is possible that in a number of species the carpel-structure may also dictate ovule shape (Endress, 1996).

### Perspectives

With this work we contribute to an emerging framework for an understanding of ovule ontogenesis (Angenent and Colombo, 1996; Reiser and Fischer, 1993) that provides an extensive basis for future investigations of ovule development and gametogenesis at the molecular level.

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