GENETIC ANALYSIS OF OVULE DEVELOPMENT

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ABSTRACT

Ovules are the direct precursors of seeds and thus play central roles in sexual plant reproduction and human nutrition. Extensive classical studies have elucidated the evolutionary trends and developmental processes responsible for the current wide variety of ovule morphologies. Recently, ovules have been perceived as an attractive system for the study of genetic regulation of plant development. More than a dozen regulatory genes have now been identified through isolation of ovule mutants. Characterization of these mutants shows that some aspects of ovule development follow independent pathways, while other processes are interdependent. Some of these mutants have ovules resembling those of putative ancestors of angiosperms and may help in understanding plant evolution. Clones of several of the regulatory genes have been used to determine expression patterns and putative biochemical functions of the gene products. Newly constructed models of genetic regulation of ovule development provide a framework for interpretation of future discoveries.

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INTRODUCTION

Function and Basic Structure of Ovules
As the precursors to seeds, ovules play a central role in sexual reproduction of angiosperms and gymnosperms, the currently dominant groups of land plants. Seeds represent a stable, readily dispersed propagule, which commonly includes stored materials for rapid and efficient establishment of seedlings. These properties result in part from the fact that seeds consist of a cooperative association between three plant generations—the parental sporophyte, the gametophyte, and the progenal sporophytes (embryo and endosperm). Evolution of this cooperation between generations, and hence evolution of the ovule, was the key event in the origin of seed plants.

The clear agronomic and evolutionary importance of ovules led to extensive study of ovule structure (reviewed in 5). Ovules of angiosperms contain only three or four morphologically distinct structures. The nucellus is the terminal region of the ovule and is the site of embryo sac formation (Figure 1a). Surrounding the nucellus are one or two integuments, lateral structures that usually tightly encase the nucellus (Figure 1a,b). The integuments are not fused at the apex of the nucellus but have an opening, the micropyle, through which a pollen tube can gain access to the embryo sac. The basal part of the ovule is the funiculus, a supporting stalk that attaches the ovule to the placental region within the carpel.

Recently, because of its relative morphological simplicity, the ovule has been perceived as an attractive structure for the study of regulation of morphogenesis. Despite this simplicity, ovule development embodies nearly all of the processes that characterize plant development in general: primordium initiation, directional division and cell expansion, asymmetric growth, and cellular differentiation. Thus, an understanding of ovule development has the potential to illuminate many aspects of plant development.

Ovule Evolution
While ontogeny does not actually recapitulate phylogeny, information on evolution of a structure can often contribute to understanding morphogenesis. Seeds (and hence ovules) are first observed in the fossil record in the Upper Devonian or Lower Carboniferous, approximately 330 to 350 million years ago. Fossils
Figure 1  Scanning electron micrographs of wild-type and mutant Arabidopsis ovules. (a) Wild-type ovules at the time of integument initiation; (b) wild-type ovules at anthesis; (c) ant-5 ovules at anthesis; (d) bel1-4  ovules showing the most common phenotype (lower right) and a carpelloid ovule (co); (e) ino at anthesis; (f) ats at anthesis; (g) sin1-1 at anthesis; (h) sup-5 at anthesis. Bars = 20 µm in all panels. c, Collar of tissue; f, funiculus; ii, inner integument; ils, integument-like structure; m, micropyle; n, nucellus; oi, outer integument; s, stigma; si, single integument. Photos are courtesy of K Robinson-Beer (a, b, d, and e) and JM Villanueva (f) or are reproduced from References 14 (g) or (40) (h), with permission.
from this time period show a series of putative evolutionary intermediate forms that suggest the origin of the first integument. These fossils show fusion of appendages (telomes or sporangiophores) surrounding a megasporangium (or megasporangiothore) to form a sheathlike integument (1, 17). Ovules of such plants were erect, had clearly defined micropyles, and closely resembled ovules of many extant gymnosperms. The current interpretation is that the first integuments originated directly from fused appendages and not from modification of leaves; in fact, leaves are also hypothesized to have derived from fusion of telomes (for example, see 16).

The details of evolution of angiosperms from their gymnospermous predecessors remain largely obscure because of major gaps in the fossil record. Despite this, several firm conclusions can be drawn regarding the evolution of the angiosperm ovule. Extant and fossil gymnosperms have unitegmic (single integument) ovules (16, 49). Bitegmy is the primitive morphology within the angiosperms, because it is the primary condition in all putatively basal groups (5; CS Gasser, unpublished information). Unitegmy is clearly a derived state that has arisen several times within the angiosperms and is largely confined to crown groups within several of the larger clades (5). Thus, the presence of a second integument is a key character separating ovules of angiosperms and gymnosperms.

The second integument has often been discussed as deriving from a cupule, a structure surrounding one or more ovules; the cupule is found in a number of fossil gymnosperms (16, 48, 49). While firm evidence supporting a relationship between the cupule and the outer integument is lacking, it is clear that the origin of the second integument occurred sometime in the Upper Jurassic or Lower Cretaceous, close to 200 million years after the origin of the first (and likely inner) integument.

Ovule Development

The development of a seed is a continuous process. For this discussion we consider ovule development to be those processes occurring prior to fertilization, with further development constituting seed development. Angiosperm ovule development was comprehensively reviewed by Bouman (5) and has also been the subject of more recent reviews (2, 38). A brief summary is presented here.

The sites of ovule initiation are referred to as placentas and are at various locations within the carpels, depending on the species. Ovules originate subderrmally through periclinal divisions within the L2 or L3 layers of the placenta. Ongoing division of these cells, in combination with anticlinal divisions in the epidermis, results in formation of an ovule primordium. One or two integuments are initiated from the chalaza, commonly in a ring of cells around the circumference of the primordium (Figure 1a). The inner integument is usually
initiated first and is almost invariably of dermal (L1) origin (5). The outer integument can be of dermal or subdermal (L2, or L2 and L3) origin (5). The integuments grow around and are tightly appressed to the nucellus and to each other. In the majority of angiosperms, asymmetric growth of the funiculus, the chalaza, the nucellus, or the integuments, or a combination of these structures, results in curvature of the ovules such that the micropyle is adjacent to the funiculus (Figure 1b).

Concomitant with the above processes, a subdermal cell within the nucellus differentiates to form an enlarged archesporial cell. A megaspore mother cell will differentiate directly from this cell or from a mitotic product of this cell (5). Four megaspores result from meiosis of the megaspore mother cell and, depending on the species, one, two, or all four will go on to form the megagametophyte or embryo sac. Most commonly, the embryo sac derives from a single megaspore. Subsequent cell divisions often produce eight nuclei separated into seven cells, but there are many species with a different cellular constitution (for reviews see 38, 53).

GENETIC ANALYSIS AND MOLECULAR CLONING
Recently, a number of groups have initiated classical and molecular genetic analysis of ovule development. This research has primarily focused on Arabidopsis thaliana and Petunia hybrida. Arabidopsis has the advantages of relatively well-developed genetic tools and extensive information on flower development. A large body of existing molecular work and a very simple transformation system make petunia a useful system for many types of molecular genetic studies. In this section, we use progression of ovule development as a framework to describe results of such genetic studies that provide a useful extension of prior morphological and anatomical studies.

Ovule Initiation and Identity
One would expect mutants in ovule initiation to lack ovules or to have a dramatic alteration in ovule placement or number. To date, such mutants have not been described. In contrast, tobacco mutant plants and transgenic petunia plants have been described that exhibit apparent alterations in ovule identity.

TOBACCO OVULE MUTANTS Two tobacco mutants (MGR3 and MGR9), which may be defective in ovule identity, were regenerated from cultured cells selected for resistance to a polyamine biosynthesis inhibitor (13, 28). The ovaries of both mutants produced apparently normal ovules and also style- or carpel-like structures in place of some ovules (13). It is possible that these mutants are defective in a function that promotes ovule identity. Both mutants had elevated levels of polyamines, but the relationship between ovule defects and
the polyamine phenotype remains unclear, as further work on these lines has not been published.

ECTOPIC EXPRESSION OF AGAMOUS To learn more about the function of AGAMOUS (AG) genes, Mandel et al (29) produced transgenic tobacco plants ectopically expressing Brassica napus AG (BAG) under control of the cauliflower mosaic virus (CaMV) 35S promoter. A significant fraction of the resulting plants exhibited floral abnormalities including conversion of sepals to carpel-like structures that develop ectopic ovules, and conversion of petals to stamens (29). These features are similar to those observed in APETALA2 (AP2) mutants of Arabidopsis (7, 23). Within the gynoecium, a subset of ovules of the transgenic plants are converted to style-like structures (29). This phenotype closely resembled that observed by Evans et al (12) in their tobacco mutants. This indicates that ectopic AG expression can cause ovule primordia to deviate from their normal developmental fate. As discussed below, however, ectopic expression of AG genes in other species can produce different results.

FLORAL BINDING PROTEINS 7 AND 11 Floral binding proteins (FBP) 7 and 11 are encoded by petunia cDNAs that were isolated on the basis of homology to the MADS box domain common to several genes that regulate flower development (3). These proteins share 90% amino acid identity with each other (3). Interestingly, both appear to be single copy genes in allotetraploid Petunia hybrida even though both are present in each of the ancestors of this species (3). RNA blot analyses and in situ hybridizations showed that the two genes are expressed in similar patterns with expression confined to the gynoecium (3). Initial expression is in the cells at the center of the flower that will give rise to the placenta. Expression persists in the developing placenta but is later confined to the ovule primordia and then to the funiculus and emerging integuments (3). In mature ovules, expression is strong in the endothelium (3).

Transgenic petunia plants were generated in which expression of both FBP7 and FBP11 was reduced because of the presence of the transgene (3). In homozygous progeny of one such co-suppression line, both FBP7 and FBP11 mRNAs were undetectable. Ovaries of these plants contained a few sterile ovules with wild-type morphology, but the majority of ovules were partially or completely converted to style-like structures (3; Figure 2b). These structures emerged directly from the placenta, not from any visible ovular structure (3). The style-like organs closely resemble those observed in the tobacco mutants of Evans et al (13) and in tobacco plants ectopically expressing BAG (29). A similar, but much weaker phenotype was observed in plants hemizygous for the transgene. The levels of mRNA of putative petunia orthologs of AG were slightly higher than wild-type levels in ovaries of homozygous plants (3).
Effects of ectopic expression of FBP11 were examined by generating transgenic petunia plants with this coding region under control of a CaMV 35S promoter (10). One primary transformant with an extreme phenotype had alterations in sepal development that included the absence of trichomes and the formation of placenta-like tissues bearing ovules at fusion points of the sepals (10). Rarely, ovules were also observed on the abaxial side of the tubular corolla, which showed no other apparent alterations (10; Figure 2c).

The carpelloid ovule phenotype of plants with suppressed expression of FBP7 and FBP11 implicates at least one of these genes as an important determinant of identity of ovules or of the placenta (2, 3). The reduced effect of the transgene in hemizygous versus homozygous plants may reflect the need for a threshold of activity as a switch for selecting the developmental fate of the meristems. A role for FBP11 in ovule and placenta identity is further supported by the formation of ectopic placental regions bearing ovules on sepals, and of occasional ovules on petals, of plants ectopically expressing this gene (10). While together these results present a strong case for FBP11 and FBP7 involvement in ovule and placental identity, other interpretations of some results are possible. The coincidence of an absence of trichomes on the ovule-bearing sepals of the FBP11-overexpressing plants suggests at least partial conversion of these
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organs to carpels, rather than merely production of ectopic ovules. Conversion of sepals to carpel-like structures is also seen in AP2 mutants of Arabidopsis and in tobacco (20, 29) or Arabidopsis (30) plants ectopically expressing AG or other related MADS-box genes (MF Yanofsky, unpublished information). Because FBP11 is in the AG group of MADS-box genes (3, 32, 34), the effects of ectopic expression of FBP11 on sepals may occur because FBP11 weakly mimicks AG.

AP2  The Arabidopsis ap2 mutation was originally identified because it reduces floral organ number and causes homeotic changes in the first two whorls of floral organs (7, 23). More recently, two ap2 alleles, ap2-6 and ap2-7, were shown also to affect ovule development. Normal ovules are produced by ap2-6 plants, but filamentous structures and structures with features of both carpels and sepals also arise from the end of a funiculus or directly from the placenta (31). These aberrations suggest that AP2 may have a role in the promotion of ovule or placental identity (31). However, these mutants produce many normal ovules, and other strong alleles of ap2 produce only morphologically normal ovules (19). AP2, is therefore, not essential for ovule development; the precise role of this gene in ovule development remains unclear.

Integument Initiation, Identity, and Development

The first morphological change to occur in the initially featureless ovule primordium is the emergence of the integuments. Extension of the integuments results from anticlinal cell divisions in combination with directional cell elongation. A number of Arabidopsis mutants have been identified that affect initiation, identity, or development of integuments.

ANT  As shown in Figure 1c, strong aintegumenta (ant) mutants fail to develop integuments (4, 11, 21). Even in putative null alleles, elimination of the integuments is not always complete, and some expansion of the chalazal region may occur late in ovule development (4, 11, 21). This expansion is at least partly under control of other genetic loci, as the degree of expansion varies in different genetic backgrounds (11). Strong ant mutants also fail to form embryo sacs (4, 11, 21).

Several weaker alleles of ant have been described (4, 21). The ant-8 allele forms a single asymmetric structure in place of the two integuments (4). This structure can grow to resemble an outer integument that partially encloses the nucellus. The ant-3 allele has even more extensive integument growth and forms two integuments, but the separation between the two integument primordia is less distinct than in wild type (21). Initiation of the inner integment is asymmetrical, and the integuments do not grow to their normal size. In rare cases a functional embryo sac develops in ant-3 ovules (21).
Klucher et al (21) showed that the early expression of the \textit{BEL1} gene (see below) was not altered in \textit{ant} mutants. Because \textit{BEL1} expression specifically marks the chalazal region of the ovule, this observation shows that a proximal-distal zonation of the ovule primordium occurs in \textit{ant} mutants. Baker et al (4) noted that cells on the surfaces of the nucellus, chalaza, and funiculus of \textit{Ant}− ovules take on different shapes and textures as the primordia age. This indicates continuation of some aspects of ovule development. Together, these observations show that strong \textit{ant} mutations result in failure in integument primordia formation or enlargement rather than failure in formation of a chalazal region or an arrest of ovule development in general.

\textit{ANT} is thus necessary for normal initiation of integument primordia, for formation of two separate primordia, and for subsequent expansion of primordia, with larger amounts of activity necessary for each of these progressive steps. Whether the absence of embryo sac development is a direct effect of the \textit{ant} mutations or an indirect effect of the lack of integuments is unknown. However, the absence of \textit{ANT} expression in the nucellus during embryo sac formation (11) argues against a direct role for \textit{ANT} protein in megagametophyte development.

In addition to altered ovule development, \textit{ant} mutants exhibit a consistent decrease in the size and numbers of other floral organs (4, 11, 21). As for the effects on ovule development, these effects indicate that \textit{ant} mutations act to inhibit initiation or expansion of lateral structures.

Two groups have independently isolated clones of the \textit{ANT} gene using insertional mutants (11, 21). The \textit{ANT} protein is homologous to the AP2 protein of Arabidopsis. In addition to the presence of a two-fold repeat of a putative DNA binding domain and a conserved spacer sequence (like AP2), \textit{ANT} also includes a serine-rich region, a glutamine-rich region, and a putative nuclear localization signal (11, 21). Together, these properties make it highly likely that \textit{ANT} is a transcriptional regulator.

The pattern of expression of \textit{ANT} as determined by in situ hybridization, is largely consistent with the visible effects of \textit{ant} mutations. \textit{ANT} mRNA was found in the primordia of all floral organs early in their development and was persistent in the margins of petals (11). \textit{ANT} mRNA was present throughout ovule primordia early in their development but was restricted to the chalazal region by the time of integument initiation (Figure 3a). \textit{ANT} mRNA was also found in meristematic regions of the shoot despite a lack of phenotypic effects of \textit{ant} mutations on these structures (11). The \textit{ANT} gene does not appear to be autoregulatory as the pattern of accumulation of \textit{ANT} mRNA was similar in ovules of wild-type and \textit{ant-9} (apparently null) mutant plants (11).

Analysis of \textit{ant-9 ap2-2} double mutant plants revealed strong synergism between these two mutations. Strong \textit{ap2} mutations led to decreased numbers of organs in the first three whorls of flowers and caused homeotic transformations
of organs that do develop in the first two whorls (8). All first, second, and third whorl organs are usually absent from flowers of double mutant plants, and such flowers consist of only a gynoecium and occasional subtending rudimentary filaments or bract-like organs (11). Thus, the effects of ant and ap2 mutations on organ number are additive. This observation, and the homology between these two genes, suggest that each gene may partially compensate for the absence of the other in promoting floral organ initiation and growth. The ovules of the double mutant plants do not differ significantly from those of ant single mutants, indicating that AP2 cannot compensate for loss of ANT function in ovule development.

**BEL1** Phenotypic effects of bell (bell) mutations are confined to the ovules where integument identity appears to be largely lost. Significant growth does occur at the chalazal regions of Bell^- ovules in the form of a single relatively amorphous collar of tissue [the “integument-like structure” (ILS); Figure 1d] (31, 40). While the asymmetric shape of the ILS primordium resembles that of a normal outer integument, subsequent growth is irregular, and the ILS does not resemble either integument. Embryo sac development rarely proceeds beyond the formation of megaspores in bell mutants, and further steps are aberrant when they occur (17, 31, 40). Growth of the ILS is not always evenly distributed, and a variable number of protuberances often extend from the edges of this structure (17, 31, 40). While the cells of the protuberances are usually
parynychmatous, a subapical cell can take on the appearance of a megasporocyte (17). Herr (17) has hypothesized that these protuberances may represent ectopic nucelli.

While the formation of a collar-like ILS is the terminal condition for the majority of Bel1− ovules, the ILS of a significant subset of ovules can expand dramatically forming carpelloid structures (31, 36; Figure 1d). These structures can include ovary and stylar regions, stigmatic regions, and secondary ovules (which reiterate the Bel1− phenotype; 31, 36).

Reiser et al (39) cloned the BEL1 gene using a T-DNA (transferred DNA of Agrobacterium tumefaciens) tagged line. The deduced BEL1 protein includes a homeodomain DNA-binding motif and is therefore likely to be a DNA-binding transcriptional regulator. The sequence of BEL1 differs significantly from that of most previously described homeodomain proteins, thus BEL1 represents a member of a new class of such proteins (39). Within flowers, BEL1 mRNA is found exclusively in ovules. BEL1 mRNA is initially present throughout an ovule primordium but becomes restricted to the chalazal region before emergence of the integument primordia. Thus, the pattern of BEL1 expression (like that of ANT) demonstrates that the chalazal domain is established before emergence of the integuments (39) and is a molecular marker for this region.

The ILS of a Bel1− ovule has been interpreted as replacing only the outer integument, with the inner integument being absent (31, 36, 40). Thus, like ANT, BEL1 may be necessary for initiation of the inner integument. BEL1 would then have a different role in the outer integument—directing it to its normal developmental fate (39). However, it is also possible that the ILS derives from cells that would normally give rise to both integuments and thus would represent a fusion of these two structures (15). In this model, BEL1 has a single role—determining the identity of the region giving rise to both integuments.

Ray et al (36) focused on the homeotic conversion of integuments to carpels late in development of Bel1− ovules. They observed that the expression of AG, a gene closely associated with carpel development (7), appeared to be higher in late stage Bel1− ovules than in wild-type ovules. In addition, they found that Arabidopsis plants containing a transgene that should lead to overexpression of AG had ovules similar to those of bel1 mutants. On the basis of these observations, they hypothesized that the carpelloid nature of bell ovules resulted from ectopic AG expression and that BEL1 was a negative regulator of AG. Subsequently, Reiser et al (39) showed that levels and distribution of AG mRNA were unaltered in Bel1− ovules early in development when the mutant phenotype was first manifest, indicating that if such negative regulation exists, it must be indirect. On the basis of his observation of putative ectopic nucelli on some Bel1− ovules, Herr (17) hypothesized that the bell mutation may be
atavistic, converting ovules to structures resembling unfused sporangiophores homologous to precursors of the first integuments.

Both these models, as well as other discussions on this gene (31, 39, 40), include the concept that \textit{BEL1} plays an important role in determining integument identity. In fact, it is possible to explain all these phenotypes if \textit{BEL1} simply directs the meristematic cells in the chalazal region to form integument primordia. In this model, the chalazal cells maintain their meristematic properties and continue to divide and expand under control of \textit{ANT} and possibly other genes. \textit{BEL1} activity causes this growth to be directed toward integument formation. In the absence of \textit{BEL1} activity, this region has three possible fates. The most common is simple maintenance of the undifferentiated state, producing the parynchymatous L.S of most \textit{Bel1}− ovules. Less frequently, this region reverts to the identities of the meristematic regions from which it has derived—either the placenta, where it then gives rise to ectopic ovule primordia, or the central floral meristem, where it gives rise to carpels. In the absence of the strong directive influence of \textit{BEL1}, there may be a delicate balance between these three fates, in which stochastic deviation from the undifferentiated state leads to a self-reinforcing commitment to the carpel primordium or placental developmental pathway.

\textit{INO} Effects of the Arabidopsis \textit{inner no outer} (\textit{ino}) mutation appear to be confined to the outer integument where both organ initiation and subsequent development are affected (4, 14, 45). Following normal initiation of an inner integument, the outer integument of an \textit{Ino}− ovule appears to initiate on the opposite side from that of wild-type ovules (4). The rotation appears to be specific to the outer integument primordium because other bilaterally symmetrical aspects of ovule development are unaltered. The funiculus bends in the normal direction toward the base of the pistil, and the nucellus bends normally toward the apex (stigma) of the pistil (4). Thus, the effect of the \textit{ino} mutation on initiation of the outer integument appears to be a 180° displacement of the region producing this structure around the axis of the ovule primordium.

The aberrantly oriented outer integument primordium of \textit{ino} mutants undergoes minimal further development following initiation (Figure 1e). \textit{INO} may have two roles, orientation of the outer integument primordium and promotion of growth of this structure. Alternatively, \textit{INO} may only affect orientation of the outer integument primordium, and the absence of further development may be a secondary effect of misorientation.

\textit{ALTERED TESTA SHAPE} In ovules of the Arabidopsis \textit{altered testa shape} (\textit{ats}) mutant ovules, the inner and outer integuments are replaced by a single integumentary structure (25). In wild-type ovules, the inner and outer integuments consist of three- and two-cell layers, respectively. The innermost layer of the
inner integument differentiates to form an endothelium (40). In mature seeds of Arabidopsis, the external layer of the outer integument produces columellae, characteristic central elevations in the desiccated cells (25). The integument of an \textit{ats} ovule consists of only three cell layers that include both an inner endothelium and an outer layer that will form columellae (25). Thus, Ats- ovules have a single integument with properties of both inner and outer integuments.

One interpretation of this phenotype is that \textit{ats} ovules fail to form the furrow separating the two integuments (25). \textit{ats} integuments remain fused together, but cell layers within the compound structure maintain their identities and differentiate appropriately.

\textbf{SIN1} The Arabidopsis \textit{short integuments 1 (sin1)} mutation affects growth of the integuments and general growth of the plant. \textit{sin1} was originally identified in an \textit{erecta (er)} background where it resulted in reduced apical dominance, short internodes, late flowering time, reduced pollen production, and infertile ovules with short integuments as a result of reduced cell elongation (Figure 1g; 24, 40). Sin1- plants are infertile because meiosis does not occur (40). Lang et al (24) found that in an \textit{ER} background \textit{sin1} internodes were of normal length (\textit{SIN1} \textit{ER} plants have internodes of intermediate length), and normal pollen production was restored. The majority of ovules of \textit{sin1 \textit{ER}} plants have short outer integuments, inner integuments much longer than in wild type, and arrested megasporogenesis. Following pollination, some ovules develop a normal outer integument, but the inner integment enlarges into a hollow structure that can be even larger than a normal seed. No morphological changes were observed after pollination of the \textit{sin1 \textit{ER}} ovules (24). Several publications (24, 35, 37, 40) provide more details on these and other aspects of the Sin1- phenotype.

The above data indicate that ER can mask the effects of \textit{sin1} on internode elongation, and that SIN1 can partially compensate for the absence of ER (24). Thus, while both ER and SIN1 can contribute to internode elongation, ER is more critical for this process. Effects of \textit{er} mutations on ovules are only visible in a \textit{sin1} background, indicating that SIN1 can completely compensate for absence of ER in developing ovules. One of several possible explanations for these effects is that SIN1 and ER are similar proteins and that their different effects reflect levels of expression of one or the other gene in specific structures.

\textbf{SUP} Arabidopsis \textit{superman (sup; also referred to as floral mutant 10, flo10)} mutants were originally identified by their effects on gross floral morphology. Sup- flowers have supernumerary stamens and a corresponding reduction in the amount of carpel tissue, sometimes leading to a complete absence of a gynoecium (6, 47). Gaiser et al (14) noted that Sup- ovules are aberrant. Formation of
the asymmetric outer integument primordium is normal in Sup− plants. However, subsequent growth is approximately equal on all sides of the primordium, resulting in a long tubular outer integument (Figure 1h). The radially symmetrical inner integument is visible in ovules of sup ino double mutants and does not appear to be affected by sup mutations (14). The specific role of the SUP gene in ovule development thus appears to be to suppress growth of the outer integument on the adaxial side of the ovule (14).

Effects of sup mutations on stamen number and carpel development have been shown to largely or completely result from expansion of expression of a floral homeotic gene, APETALA3 (AP3), outside the third whorl of primordia and into the region normally giving rise to the gynoecium (7, 44, 47). Ovules of sup ap3 double mutants are indistinguishable from those of sup single mutants (14). This indicates that AP3 does not play a role in the effect of sup mutations on ovule development and that there are significant differences between the mechanisms by which SUP mediates floral and ovule development.

Cloning and sequencing of the SUP gene showed it to encode a protein with properties of a DNA-binding transcription factor (44). SUP mRNA was detected in the innermost region of the third whorl of floral organs, and in the funiculus adjacent to the outer integument, but was not detected in the fourth whorl primordia, or in the integument primordia—the structures most affected by the mutation (44). One simple mechanism that could explain both apparently non-cell-autonomous effects of SUP is that the function of the SUP gene product is to create a boundary that prevents expansion of the zone of expression of some factor beyond the region of cells where SUP is present. In the floral apex, this factor could be AP3, while an as yet unknown growth-promoting factor would be regulated by SUP in the outer integument of ovules (44).

TOUSLED The tousled (tsl) mutation was originally identified by its floral aberrations (43). This mutation leads to a decrease in the number of organs in the three outer whorls, a slight increase in the number of carpels in the fourth whorl, and altered morphology of all floral organs (42). The Tsl− gynoecium exhibits reduced development in apical tissues, leads to failure of postgenital fusion of the style and septum (42). Tsl− ovules have a protruding inner integument as a result of abnormal elongation of this structure, and variable but usually reduced development of the outer integument (42). The opposite effects of TSL activity on growth of the gynoecium and the inner integument indicate that the serine/threonine kinase activity of this protein (41, 43) regulates different aspects of cell proliferation in different plant structures.

LEUNIG The leunig (lug) mutation was identified by its pleiotropic effects on leaves and floral organs (22, 26). Lug− plants have narrow serrated leaves,
slightly carpelloid sepals, and stamenoid petals. Genetic experiments indicate that a primary role of LUG may be to negatively regulate expression of \textit{AG} and that aberrant \textit{AG} expression may be responsible for many of the effects of the \textit{lug} mutation (26). Lug\textsuperscript{−} and Tsl\textsuperscript{−} plants have similar gynoecia and ovules, with ovules of both having a protruding inner integument (42, 45). The observation that ovules of \textit{tsl lug} double mutants have similar phenotypes to either single mutant suggests that both genes could act on a single process in ovule development (42). The effects of \textit{lug} mutations on ovules are distinct from the phenotypes observed in plants overexpressing \textit{AG}; it is unlikely that \textit{AG} is responsible for the Lug\textsuperscript{−} ovule phenotype.

\textbf{OTHER MUTANTS} In their recent analysis of a large set of Arabidopsis ovule mutants, Schneitz et al (45) provide initial descriptions of six new mutations affecting integument development. In \textit{huellenlos (hll)} mutants, integuments appear to initiate, but their development is limited. The inner integument primordia undergo only a few cell divisions, and the region from which the outer integument would form usually undergoes only minimal cell expansion and no cell division. This lack of development can be followed by precocious degeneration of the nucellar region. The \textit{unicorn (unc)} mutation acts relatively early in ovule development and leads to formation of a protrusion at the base of the outer integment. Other mutations act later in development and lead to dissected outer integuments (\textit{strubbelig, sub}), highly irregular integuments (\textit{blasig, bag}), integuments with enlarged cells (\textit{mollig, mol}), or a protruding inner integment (\textit{laelli, lal}). Further characterization of these mutations will allow more complete understanding of their roles in ovule development. Many additional genes likely remain to be identified.

\textbf{Embryo Sac Development}

As noted above, several ovule mutants affect both the sporophytic parts of ovules and the embryo sac. Strong alleles of \textit{bel1, sin1}, and \textit{ant} lead to early arrest of embryo sac development (4, 11, 21, 40). The absence of detectable expression of \textit{ANT} or \textit{BEL1} in cells giving rise to the embryo sac led to the hypothesis that failure in embryo sac formation may be an indirect result of the absence of integuments (11, 21, 39). The recently described \textit{hll} mutation, which results in highly reduced integuments, also fails to form an embryo sac (45). \textit{ats} and \textit{ino} mutants produce ovules that have one integument around the nucellus, and both mutants produce at least some functional embryo sacs (4, 25). \textit{ats ino} double mutants have a naked nucellus and fail to form embryo sacs (4). In every case in which integuments do not enclose the nucellus, an embryo sac fails to form, and at least one integument may be essential for normal embryo sac formation. It is clear that a sheathing integument is not sufficient for this
process, however, as numerous mutants have been described that have aberrant embryo sacs despite the presence of normal integuments (18, 45, 51).

MODELS FOR GENE ACTION

A number of conclusions about the regulation of this process can be drawn from observations of wild-type ovule development (31, 40, 46). The regular arrangement of ovules on the placenta demonstrates the existence of a patterning process to define the locations for ovule initiation. After initiation of an ovule primordium from the placenta, proximal-distal patterning of the primordium defines three zones that will give rise to the funiculus, integuments, and nucellus (46). In addition, the bilateral symmetry of most ovules indicates that lateral patterning must also occur at this time (4). Ovule mutants will aid characterization of genetic interactions to formulate more detailed models of regulation of ovule development.

Ovule Genes and the ABC Model

Two groups have attempted to interrelate regulation of ovule development with the now well-established “ABC” model of floral organ identity (9, 27, 33, 52). As noted above, the simple model of Ray et al (36), in which $BEL1$ acts directly as an inhibitor of $AG$ action in ovules, was not supported by more recent molecular studies (39). Transgenic petunia plants under- or overexpressing FBP11 show an apparent loss of ovule identity and formation of ectopic ovules, respectively (3, 10). Based on these observations, and the fact that the petunia placenta arises from the floral meristem, it was proposed that FBP11 is a member of a “D” class of genes regulating placenta and ovule identity (2, 10). Because no specific interactions between D genes and ABC genes are proposed, additional work will be required to see whether this model adds to our understanding of ovule and placental development.

Ovule Gene Interactions

In contrast to the ABC classes of genes regulating floral organ identity, where numerous different interactions were found (9, 27, 33, 52), interactions among ovule genes have primarily been shown to be either strict epistasis or simple additivity. With respect to effects on ovules, $ant$ was found to be epistatic to $bell$ (4, 21), $ap2$ (11), $ino$ (4), $sin1$ (4), and $sup$ (4). $bell$ was shown to be epistatic to both $ino$ (4) and $sup$ (14), and $ino$ was shown to be epistatic to $sup$ (4). These relationships imply that these genes act in a common developmental (but not necessarily biochemical or signal transduction) pathway, and help to define their order of action (see below). The epistasis of $ant$ over all these mutations is easily explained by the fact that they affect the integuments, which are absent in $ant$ mutants.
While ant was epistatic to sin1, sin1 showed apparent simple additivity with bel1 (40), and ino (4). The additivity with bel1 and ino may indicate that the effects of sin1 are relatively independent of the actions of these other two genes. Because ino simply eliminates the outer integument, it is not surprising that the effects of sin1 on the inner integument are still readily apparent in the double mutant. That sin1 has an effect on the ILS of bel1 mutant ovules is somewhat more surprising. Most other evidence indicates that the ILS forms as a result of loss of integument identity. However, the fact that sin1 still has an effect on this structure is an indication that the loss of integument identity may not be complete. Additive effects with several different ovule mutations may be an indication that SIN1 has a general role in cellular function and that its effects on integument development may be due to its pattern of expression. That sin1 mutations can also have effects on elongation of other parts of plants is also consistent with this hypothesis.

The ino and ats mutations also show additive effects (4). In double mutant plants, the aborted outer integument (resulting from the ino mutation) is fused to the inner integument (the result of the ats mutation) supporting the hypothesis that ats causes integument fusion (4). It further appears that the fusion to the abortive outer integument prevents full development of the inner integument, and the nucellus remains uncovered. As noted above, this absence of a sheathing integument is associated with failure in embryo sac development (4).

**Comprehensive Models of Ovule Development**

In their recent review, Angenent & Colombo (2) integrated information from studies on Arabidopsis and petunia to describe ovule development as a linear series of steps, and associated a total of seven genes with regulation of specific steps. According to their model, FBP7 and FPB11 participate in ovule initiation, ANT participates in integument initiation, BEL1 participates in integument identity, INO and SIN1 participate in integument elongation, and SUP participates in asymmetric proliferation.

On the basis of new and previous analysis of ovule mutants, Baker et al (4) propose a similar order of gene action in a more complex, branched model for genetic regulation of ovule development. In this model, following the patterning of the ovule primordium into the funiculus, chalaza, and nucellus, each of these regions develops in at least partial independence of the others. The Arabidopsis ovule genes described to date are proposed to act primarily within the chalazal region, where they govern the development of the integuments. Origination of the integument primordia as two separate structures is viewed as a specific genetically regulated event under the control of ATS, ANT, and BEL1. The pathway branches further with separate developmental pathways for the inner and outer integuments, and for cell division and cell expansion in
these structures. Effects of the ovule mutations on embryo sac development are proposed to be indirect, resulting from the absence of sheathing integuments (as also proposed by others for \textit{BEL1} and \textit{ANT} \textsuperscript{11, 21}).

Schneitz et al. \textsuperscript{45} independently formulated a branched model of regulation of ovule development that shares many features with that proposed by Baker et al. \textsuperscript{4}. The model includes branches to indicate the relative independence of development of different parts of ovules but parses the overall process into types of developmental processes (pattern formation, initiation of morphogenesis, and morphogenesis) rather than into ovule-specific developmental events. This model also incorporates additional genes newly identified by the authors \textsuperscript{45}.

Using information from all the above models, we propose a consensus model representing the current state of knowledge of the order of action and roles of genes known to be involved in ovule development (Figure 4). The model describes Arabidopsis ovule development because most currently identified genes are from this species. As in other models, we hypothesize that there may be ortholog(s) of the petunia FBP7/FBP11 genes in Arabidopsis that play similar roles in this species. The model also includes establishment of a lateral pattern, necessary for the bilaterally symmetrical aspects of ovule development, which was not addressed in previous models.

**OVULE GENES, FLOWER GENES, AND PLEIOTROPIC EFFECTS**

As noted in the detailed descriptions of the genes affecting ovule development, mutations in a number of these genes result in pleiotropic effects, with many of these effects confined to flowers. In some cases, enough information is available to provide a mechanistic explanation for the pleiotropy. For example, the data on sequence similarity and overlapping expression patterns for \textit{ANT} and \textit{AP2} provide a reasonable explanation for the partial functional redundancy of these genes in floral organ formation \textsuperscript{11, 21}. This redundancy raises questions about the “original” role of \textit{ANT}, the origin of \textit{AP2}, and the role of their common ancestor gene. Because ovules precede flowers in the ancestors of angiosperms, one possibility is that the earliest role of \textit{ANT} was in promotion of integument formation. \textit{AP2} could be a diverged duplicate of \textit{ANT} that evolved to its current role during the evolution of flowers. Because Arabidopsis contains a large family of other related genes, \textit{AP2} could also derive from another member of this family that had a role in other aspects of development in the ancestors to angiosperms. \textit{lug} mutations also appear to interact synergistically with \textit{ap2} mutations, but \textit{lug} has entirely different effects on ovule development than does \textit{ant}. The effects of \textit{lug} mutations on ovules are, however, not as
Figure 4 Integrated model of genetic regulation of ovule development. Features of published models for regulation of ovule development (2, 4, 39, 45, 46) were assembled to summarize current knowledge of genetic regulation of ovule development. Progress of ovule development is indicated from bottom to top by black arrows, and intermediate structures of wild-type ovule development are shown unshaded. Gene designations are adjacent to the process they are believed to affect. Parentheses indicate greater uncertainty in placement. (?, Predicted genes that have not yet been identified. Shaded structures represent phenotypes at anthesis of ovules of mutants in each of the indicated genes. Bars at the right of the figure relate the pathway to developmental process descriptions of Schneitz et al (45).
profound as those of ant mutations, and thus LUG may play a less essential and more recently derived role in ovule development. The dual roles of SUP in flower development and in outer integument asymmetry have been hypothesized to be two manifestations of regulation of cellular proliferation by this gene (44). Because SUP acts through AP3 in flower development and not in ovule development, it is also possible that two different mechanisms are involved in these processes. It is unknown whether evolution of integument asymmetry, or even a second integument, preceded flower evolution; thus, it is not possible to tell which of the two roles of SUP is primary and which is derived.

Both tsl and sin1 mutations have multiple pleiotropic effects on flower and plant development. TSL is a serine/threonine protein kinase (41) and thus may have impacts on a variety of different processes that can be regulated by protein phosphorylation. Plants contain many protein kinases that may differentially mask the effects of tsl mutations in different parts of plants. TSL may, therefore, play roles in plant development in addition to those indicated by the observed phenotypes. The variety of effects of sin1 on ovule, flower, and plant development indicate that, like TSL, the product of this gene may affect a variety of processes through a single mechanism. The only clue we have to the possible nature of SIN1 is its apparent partial overlap in function with ER, making it possible that these proteins are homologous or that they interact in some way. ER is proposed to be a receptor kinase that includes extracellular domains (50). If SIN1 is indeed homologous to ER, then its pattern of expression, in combination with differential presence of specific phosphorylation targets, could explain the wide range of processes affected by sin1 mutations.

OVULE GENES AND OVULE EVOLUTION

The evolution of ovules was briefly described at the beginning of this review. Because ovule development is ultimately under genetic control (although this control can manifest through other downstream mechanisms—wall tension, cytoskeletal arrangements, diffusible gradients, plasmodesmatal trafficking, etc), evolutionary changes in ovule morphology must coincide with changes in genes regulating this process. Several of the currently identified ovule regulatory genes may have been important participants in initial evolution of ovules and subsequent radiation of angiosperm ovule morphology.

Ovules of strong ant mutants completely lack integuments. In this respect, they resemble the sporangiophores of the precursors to seed plants. The essential role of ANT in formation of integuments implies that evolution of this function for this gene was concomitant with evolution of integuments. Investigation of conservation of function of ANT orthologs in other species will help to verify or refute this hypothesis.
Herr (17) interprets one of the phenotypes of Bel1− ovules as a branching axis with multiple terminal nucelli. On this basis, he has speculated that bell mutants are atavistic mimics of an even earlier evolutionary precursor to ovules—a fertile axis comprising multiple sporangiophores. However, this is only one of three fates of the ILS of bell mutants, the others being an amorphous collar of tissue or a carpelloid structure. We therefore favor the hypothesis, outlined in our description of this mutant, that the loss identity resulting from bell mutations leaves the cells in a meristematic state without a firm direction to a specific fate. In either case, the role of BEL1 in determination of integument identity argues for coincidence of evolution of this gene and evolution of integuments.

Ovules of ino mutants, which have only a single (inner) integument, resemble those of extant and fossil gymnospermous plants, including putative progenitors of angiosperms. This implicates the INO gene as a critical component in both development and evolution of the outer integument. The origin of the outer integment remains largely obscure, and molecular analysis of this gene—and of potential orthologous genes in conifers and Gnetales—will provide a new avenue to address the previously untractable question of this origin. Unitegmy is clearly a derived state in the majority of angiosperms displaying this trait (see the section on Ovule Evolution, above). The unitegmic ovules of some angiosperms appear to result from loss of the outer integument (5) and therefore represent phenocopies of ino mutants. It will be of great interest to examine INO orthologs in these species. The most common alteration leading to unitegmy is, however, congenital fusion of the two integuments into a single structure (5). This is the apparent effect of the ats mutation. Molecular studies on ATS orthologs will enable testing of the obvious hypothesis that evolution of this type of unitegmy results from alteration in the nature or expression patterns of such genes.

The asymmetric shape of ovules can be seen to result from several different genetically separable steps including curvature of the funiculus, the initial orientation and asymmetric shape of the outer integument primordium, bending of the base of the nucellus, curvature of the nucellus and embryo sac, and asymmetric expansion of the outer integument (4, 45). SUP is clearly a critical determinant in the last of these processes; this gene is likely to be involved in the evolutionary changes separating amphitropous and orthotropous ovules. Studies on the nature and expression of SUP in a variety of angiosperms may allow the determination of the molecular mechanism for some of the evolutionary changes in ovule morphology.

PERSPECTIVE
The past five years have seen rapid progress on genetic regulation of ovule development. Before this period, no systematic attempts were made to identify
genes involved in this process. Now more than a dozen loci have been identified, and ongoing efforts should uncover more. While some of these genes apparently function only in ovules, many also regulate aspects of development in other floral and even vegetative structures. In retrospect, this is not surprising given that mutations were selected for both alterations of identity of ovules and their substructures, but also for effects on ovule morphology. Thus, genes regulating general aspects of cell division and directional expansion, which would be expected to be important in morphology of all parts of plants, could be identified. Ovules provide a simple system in which it may be possible to determine the specific roles of such genes. The isolation of ovule regulatory genes has only just begun but has already produced interesting results. Studies using the cloned genes will eventually provide an understanding of regulation of ovule development at the molecular level. Of equal importance, cloned genes allow ongoing studies to cross taxonomic lines by facilitating isolation of orthologous genes from different species. Examining the presence or absence of such genes, or the nature and regulation of genes once found, may provide a new avenue for understanding the evolutionary origin of angiosperm ovules and the evolution of the wide variety of ovule morphologies within the angiosperms.

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