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Regulation of Ovule Development

Debra J. Skinner,[a,b,1] Theresa A. Hill,[a,1] and Charles S. Gasser[a,2]

[a] Section of Molecular and Cellular Biology, University of California, Davis, California 95616
[b] Genetics Graduate Group, University of California, Davis, California 95616

INTRODUCTION

Ovule development in Arabidopsis and other plants has been the focus of classic and molecular genetic analyses in recent years. This has been an exciting time to be involved in plant developmental biology, because many genetic pathways and regulatory mechanisms have been elucidated. Continued studies of ovule mutants have contributed to and benefited from these advances. Ovule development involves the same basic processes necessary for the formation of other plant organs, such as primordium initiation and specification, directed cell division and expansion, and asymmetric growth and differentiation. However, ovules differ from other plant structures in their reproductive function and apparent evolutionary origin from sporangiophores (Herr, 1995), allowing the basic developmental processes to be studied in a unique context. Researchers today are using both the wealth of botanical knowledge and new molecular insights to achieve a more comprehensive understanding of ovule morphogenesis and evolution. Recent reviews by Grossniklaus and Schneitz (1998) and Gasser et al. (1998) describe progress in the analysis of several ovule mutants. Here, we extend these earlier reviews with the latest information on the roles and nature of genes involved in the formation of the placenta as the site of ovule initiation, ovule identity, patterning of the ovule primordium, and control of integument morphogenesis. We focus primarily on recent results in Arabidopsis and indicate when results from other species are being discussed.

Ovules are the site of processes essential for sexual plant reproduction, including the formation of the megagametophyte, fertilization, embryogenesis, and finally, the formation of the persistent propagule—the seed. Arabidopsis ovules are initiated as small, finger-like primordia from regions (the placentas) of the internal surface of the carpels (Robinson-Beers et al., 1992). The inner and outer integuments arise from the surface of each ovule primordium, with their region of origin defining the chalaza, which separates the apical nucellus from the funiculus (Figures 1A and 1B). The two integuments grow to cover and enclose the nucellus, leaving a small opening, the micropyle (Figure 1C). The funiculus, or stalk, provides a conduit for nutrients to the developing ovule and embryo and partly determines the position of the micropyle. The integuments are required to house the embryo sac, contribute to ovule positioning, and later, form the protective seed coat. The nucellus provides the cellular initial for the differentiation of a megasporocyte, which undergoes meiosis and mitosis to produce a seven-celled megagametophyte, the embryo sac (Figure 1D) (Webb and Gunning, 1990; Mansfield et al., 1991).

Molecular genetic analyses with genes important for ovule development that have contributed to recent advances revealing the genetic pathways and regulatory mechanisms involved in plant development are the focus of this review.

SPECIFICATION AND FORMATION OF THE PLACENTA

Ovules derive from specialized meristematic regions within the carpels referred to as the placentas. In Arabidopsis, the mature gynoecium consists of two congenitally fused carpels whose locules are separated by a central septum. During gynoecium development, longitudinal medial ridges, several cells wide, protrude from opposite sides into the center of the cylinder formed by the elongation of the fused carpel primordia (Figure 2A) (Bowman et al., 1999). The ridges of cells fuse and give rise to the septum. Placental tissue differentiates along the length of the septum adjacent to the lateral walls (Figure 2A). The ovule primordia emerge from the placental regions during stage 9 of flower development (Smyth et al., 1990). Despite the placenta's importance in ovule development, the molecular events that lead to placental development are not yet well understood.

To understand the relationship of the placenta to the rest of the gynoecium, it is useful to consider the possible evolutionary origins of the carpels. The apparent monophyly of the seed plants (Chaw et al., 2000) and the extensive gymnosperm fossil record show that carpels arose after ovules (Stewart, 1983). Carpels are proposed to have evolved from ancestral foliar organs, either leaf-like sporophylls that folded to enclose the ovules (Cronquist, 1988) or bract-like structures that subtend shoot-like ovules (Taylor, 1991). The evolutionary origin of the placenta and the chronology of its enclosure within the angiosperm carpel are ambiguous. Several lines of evidence, reviewed by Bowman et al. (1999), support the theory that the medial ridge is an outgrowth of part of a marginal domain that would correspond to the edge of the ancestral leaf-like structure and that the placenta is part of, or has been fused to, this margin. Extensive redundancies between the pathways and genes involved in the formation of the medial region and the placenta have made genetic analysis of this region problematic.

The carpel represents a complex scenario for the patterning and maintenance of meristematic cells. The elongating and differentiating carpel primordia must maintain existing or produce

[1] These authors contributed equally to this work.
[2] To whom correspondence should be addressed. E-mail csgasser@ucdavis.edu; fax 530-752-3085.
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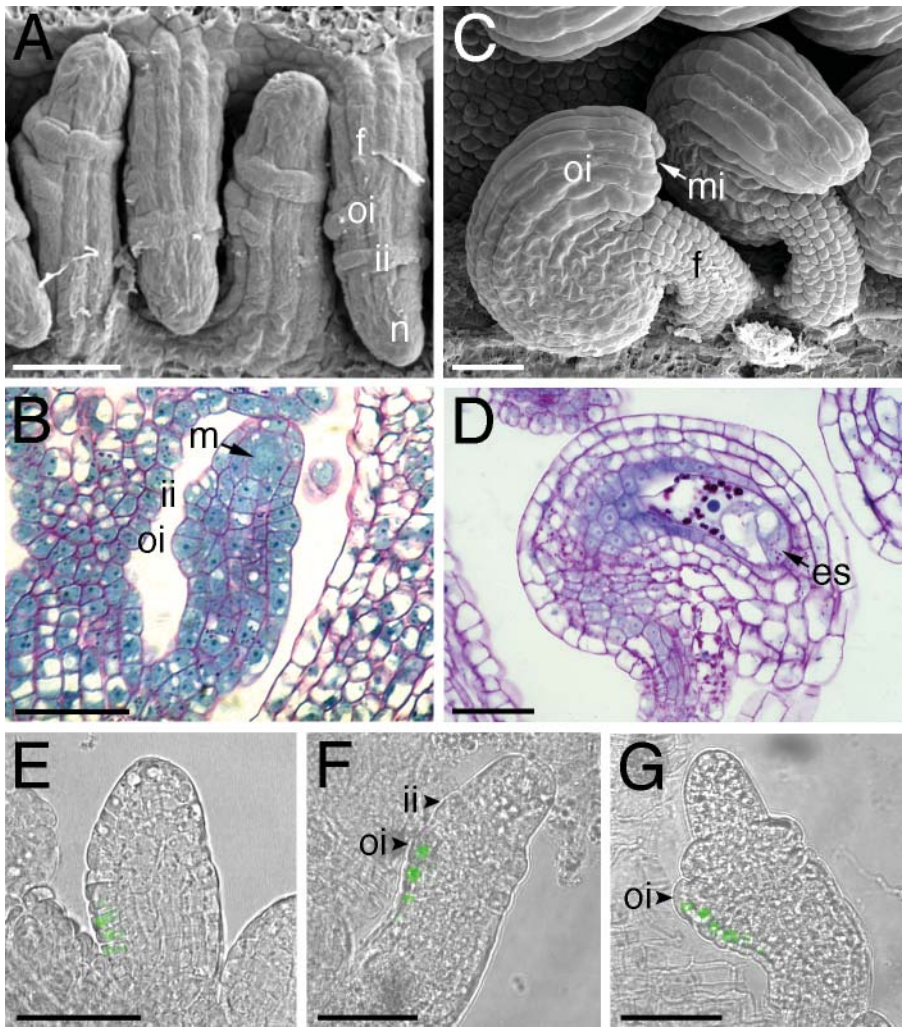

Figure 1. Arabidopsis Ovule Development.

All images are oriented with the gynobasal direction (toward the base of the gynoecium, or receptacle) to the left and the gynoapical direction (toward the apex of the gynoecium, or stigma) to the right.

(A) to **(D)** Wild-type Landsberg *erecta* ovules.

(A) Primordia at stage 11 of flower development. The first cells of the inner integument form a ring around the primordium. The outer integument is initiating on the gynobasal side of the ovule primordium, below the inner integument.

(B) Primordium at same stage as in **(A)** showing derivation of the integuments from the L1 layer and differentiation of the megasporocyte.

(C) Mature ovules at anthesis. The integuments have grown to cover the nucellus, and the funiculus has curved in the opposite direction.

(D) Mature ovule showing the cell layers of the integuments and the embryo sac.

(E) to **(G)** Composite differential interference contrast and laser confocal images of wild-type Landsberg *erecta* ovules with an INO:GFP reporter fusion protein expressed under the control of the *INO* promoter.

(E) The fusion protein accumulates in the nuclei of cells in the proximal half of the region that will give rise to the outer integument at stage 10.

(F) Fluorescence persists in this region as the integument grows in stage 11.

(G) The fusion protein is visible only in the outermost cell layer of the outer integument as it continues to grow in stage 12.

es, embryo sac; f, funiculus; ii, inner integument; m, megasporocyte; mi, micropyle; n, nucellus; oi, outer integument. Bars = 25 μm. Images in **(A)**, **(C)**, and **(D)** courtesy of K. Robinson-Beers; **(B)** courtesy of J.M. McAbee; **(E)** to **(G)** courtesy of R.J. Meister.

new meristematic regions—the placentas—that will subsequently generate ovule primordia. The medial domain of the gynoecium is thought to remain in a relatively undifferentiated state, compared with the lateral domains, while the cylinder elongates. This state is required to allow the later development of marginal tissues, including the meristematic activity of the placenta. *SHOOT MERISTEMLESS* (*STM*), *CUP-SHAPED COTYLEDONS1* (*CUC1*), and *CUC2* are expressed between incipient primordia in meristems and are known to be involved in meristematic cell maintenance (Long et al., 1996; Long and

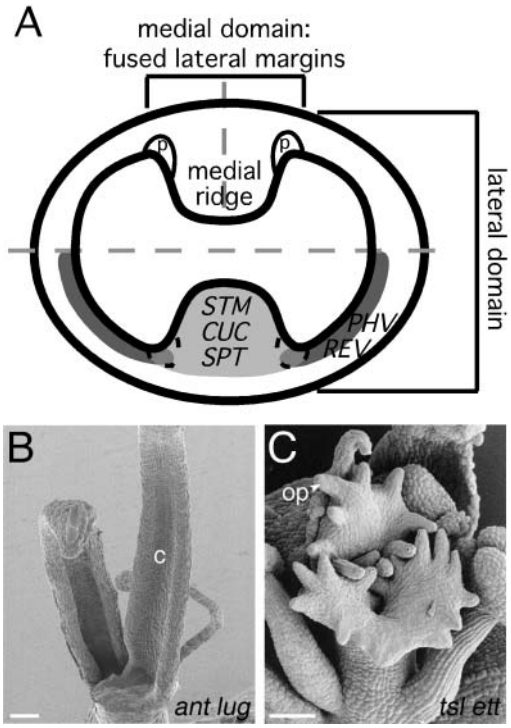

Figure 2. Development of Carpel Marginal Tissues.

(A) Scheme of a cross-section of the Arabidopsis gynoecium. Structural components are indicated at the top, and the bottom shows gene expression patterns. The medial domain is formed by congenital fusion of the lateral margins (separated by the vertical line). The internal medial region comprises both the medial ridge and the placentas (p). *STM*, *CUC1*, *CUC2*, and *SPT* are expressed in the medial region. This expression overlaps with *PHAVOLUTA* and *REV* expression in the regions from which ovule primordia will emerge, the placentas. This overlap may be an important factor in the formation of the placentas.
(B) Unfused carpel (c) of an *ant-9 lug-3* double mutant. Medial regions are lost, including placenta and ovules. Bar = 100 μm.
(C) Apparently free placentas decorated with ovule primordia (op) form in the absence of other carpel structures in the *tsl-1 ettin-2* double mutant. Bar = 50 μm.
Image in **(B)** courtesy of X. Liu; **(C)** courtesy of J.L. Roe and P.C. Zambryski.

Barton, 1998; Aida et al., 1999; Takada et al., 2001), a process recently reviewed by Clark (2001) and Lenhard et al. (2002). In the carpel, they are restricted to expression in the medial domain and are likely to maintain a meristematic state there. *AINTEGUMENTA* (*ANT*) encodes an APETALA2 (AP2) domain transcriptional regulator whose main function is to promote the outgrowth of determinate structures through cell proliferation (Elliott et al., 1996; Klucher et al., 1996; Krizek, 1999). As expected by their antagonistic roles, *ANT* expression does not overlap with *STM* expression in meristems (Elliott et al., 1996; Long and Barton, 2000). However, in the carpel, *ANT* is expressed in the medial domain with *STM*. This could be attributable to an ongoing requirement for *ANT* in the outgrowth of the carpel primordia, which is balanced with the negative effects on differentiation of *STM* in the medial domain.

In addition, *ANT* is likely to play a larger role in medial domain development, as shown by double mutant analysis with *LEUNIG* (*LUG*), a putative transcriptional corepressor (Conner and Liu, 2000). *lug* and *ant* mutants both have weak effects on marginal tissue formation, but double mutants show a strong synergistic phenotype: total loss of septum, placentas, and ovules (Figure 2B) (Liu et al., 2000). Although both of these genes are known to repress *AGAMOUS* (*AG*) genetically, this loss of the medial domain is not an effect of *AG* overexpression. Rather, *ANT* and *LUG* seem to share a vital role in promoting cell proliferation in the marginal tissues of the pistil, specifically the medial ridge. In addition, both genes show high expression in placentas and ovules that persists after carpel expression decreases (Elliott et al., 1996; Conner and Liu, 2000). Thus, in addition to other roles within the ovule, *ANT* and *LUG* also may be required specifically to promote the growth of the placenta to allow ovule primordium formation.

Other genes that may influence medial domain formation and affect the production of ovules include *CRABS CLAW* (*CRC*), *SPATULA* (*SPT*), and *TOUSLED* (*TSL*) (Roe et al., 1993, 1997a, 1997b; Alvarez and Smyth, 1999, 2002). The reduction in ovule formation shown by mutants of these and other genes can be attributed to defects in marginal regions. *CRC* also may play an additional role, however, because there is a longitudinal strand of expression in the internal region of the carpel, adjacent to the placenta, early in carpel development (Bowman and Smyth, 1999). The loss of this part of *CRC* expression is partly responsible for the reduced number of ovules observed in *crc* mutants (Bowman et al., 1999; Bowman and Smyth, 1999; Alvarez and Smyth, 2002). Thus, CRC may contribute more directly to placenta and primordium initiation, perhaps through abaxial signaling in the region or by limiting the expression of STM or other KNOX genes, which are known functions of the YABBY domain protein family (Bowman et al., 2002; Kumaran et al., 2002).

The interaction of meristem factors in the medial ridge and adaxial factors in the differentiating valve also could provide the impetus for the initiation of placental growth in a manner that is similar to the positive effect on meristem formation of adaxial identity in lateral organs (McConnell and Barton, 1998; Alvarez and Smyth, 2002). *STM*, expressed in medial domains, and *REVOLUTA* (*REV*) (and other related genes), expressed laterally, could be these factors (Figure 2A). However, it is unlikely that these factors contribute directly to identity in this region.

A double mutant of the *ettin* and *tsl* genes produces structures that could be interpreted as naked placentas: there is no evidence of carpel structures other than placenta and ovules (Figure 2C) (Roe et al., 1997b). Thus, signals provided by carpel walls may not be necessary for placental formation. This result, and the fact that placental position is particularly plastic across angiosperm species, suggests that the placenta may have evolved from an originally separate fertile structure that was recruited later onto carpel walls. However, fossil evidence corroborating this model is lacking.

Identity of Ovule Structures

Placentas and ovules are formed only within the context of carpel identity. *AG* is a potent promoter of carpel identity and, as such,

possibly promotes the formation of all structures within the gynoecium, including ovules (Bowman et al., 1989; Yanofsky et al., 1990; Ferrándiz et al., 1999). *AG* expression, initially seen throughout the gynoecium, remains high in ovule primordia and integuments (Bowman et al., 1991; Reiser et al., 1995). Although the ovules formed on the carpelloid sepals of *ap2 ag* mutants are evidence that *AG* is not absolutely required for ovule formation, the fact that there is an increase in the number of undifferentiated primordia relative to *ap2* single mutants indicates that *AG* is a contributor to ovule development. However, overexpression of *AG* homologs in *tobacco* ovules led to the conversion of ovules to carpelloid structures (Mandel et al., 1992). In Arabidopsis, ectopic expression of *AG* had little effect on ovules, whereas expression of *BAG*, the *Brassica* ortholog of *AG*, caused transformation of the integuments into carpelloid structures (Ray et al., 1994). Therefore, the designation of the primordium as an ovule requires discrimination between carpel and ovule identity. Mutants have been observed that have phenotypes similar to the overexpression of *AG*, with conversion of some ovule structures to carpel structures. These phenotypes suggest that these genes work to create or maintain the necessary balance between *AG* activity and ovule identity that allows ovule structures to develop.

BEL1 encodes a homeodomain protein known to be required for integument morphogenesis, and mutations in this gene affect ovule identity (Robinson-Beers et al., 1992; Modrusan et al., 1994; Ray et al., 1994; Reiser et al., 1995). *bel1* mutants initiate a single amorphous structure that grows as a collar around the nucellus in place of the integuments (Figure 3A). This structure has been shown to accumulate *AG* transcript at anthesis, when *AG* expression decreases in the wild type (Modrusan et al., 1994; Ray et al., 1994). In some ovules, this aberrant outgrowth continues to proliferate after anthesis and becomes carpelloid, displaying features of the ovary, style, and stigma (Figure 3B) and even producing a set of secondary ovules (Modrusan et al., 1994; Ray et al., 1994). In addition, the funiculi of *bel1* mutants continue to divide and lose their characteristic organized appearance. Because the expression of *AG* in the integuments increases only after manifestation of the *bel1* integument phenotype and at least some *AG* activity contributes to ovule identity, the mechanism for *BEL1* repression of the *AG* carpel promotion function remains unclear. It appears that the loss of integument or chalazal identity allows development to proceed along a poorly defined pathway that can sometimes default to the background pathway of carpel development.

Analysis of the phenotypes observed in a triple mutant of *ag*, *bel1*, and *ap2* expands our understanding of the function of *BEL1* and may reveal why there is a delay in the acquisition of carpel identity in the *bel1* mutant. In 1% of triple mutant ovules, there is a restoration of normal integument growth, which never occurred in *bel1* single or *bel1 ap2* double mutants (Western and Haughn, 1999). Therefore, other genes are capable of providing integument identity in the absence of *BEL1*. These genes also could act to repress carpel identity during the earlier stages, delaying the onset of the carpel phenotype in *bel1*. However, because normal integument growth is seen only when *AG* also is removed, it seems that the integument promotion functions of these genes cannot compete effectively with the carpel identity

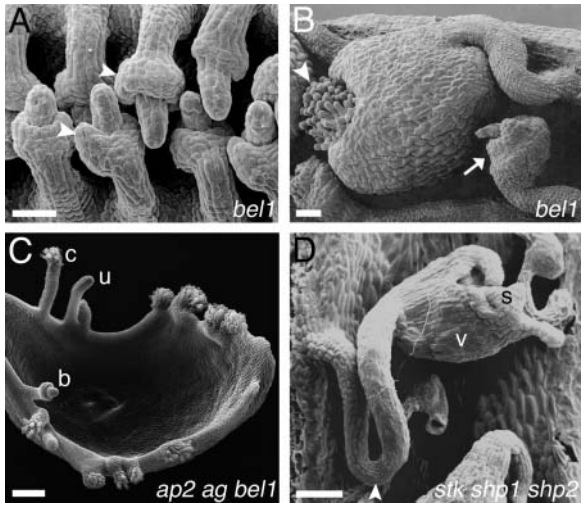

Figure 3. Ovule Identity Mutant Interactions.

(A) *bel1-1* ovules at stage 12 of flower development have an amorphous collar of cells in place of the two integuments (arrowheads).
(B) After anthesis, the collar of cells can continue to grow and become carpelloid. Stigmatic papillae (arrowhead) are visible on this secondary carpel. However, some ovules arrest growth after anthesis and degenerate (arrow).
(C) In the *ap2-6 bel1-3 ag-1* triple mutant, ovules are formed on the carpelloid sepals. These ovules usually are undifferentiated structures (u), although normal *bel1*-like (b) and carpelloid (c) ovules also form.
(D) An ovule of the *stk shp1 shp2* triple mutant after anthesis. A funiculus supports valve-like (v) and style-like (s) structures that form in the distal portion of the mutant ovule. An arrowhead marks the distal end of the funiculus.
Bars = 100 μm in **(A)** and **(B)** and 50 μm in **(C)** and **(D)**. Images in **(A)** and **(B)** courtesy of K. Robinson-Beers; **(C)** courtesy of T.L. Western and G.W. Haughn; **(D)** courtesy of A. Pinyopich and M.F. Yanofsky.

provided by *AG*. Thus, one model for the function of *BEL1* is that its primary function is to control *AG* activity, which in turn allows other genes to provide integument identity. *BEL1* also is likely to have some contribution to ovule identity, because there is an increase in the number of undifferentiated ovule primordia in the triple mutant *ag bel1 ap2* relative to *ag ap2* (Figure 3C) (Western and Haughn, 1999). These functions could be causally related: the strong promotion of chalazal identity by BEL1 may override the effect of *AG* in this region. BEL1 and other factors play a positive role in integument identity, and the absence of that role could allow AG to eventually predominate, causing the integument to become carpelloid in a self-reinforcing manner.

Recent results demonstrate that three MADS box genes that form a monophyletic clade with *AG*—*SHATTERPROOF1* (*SHP1*), *SHP2*, and *SEEDSTICK* (*STK*, also known as *AGL11*)—share a common function in promoting aspects of ovule identity (Ma et al., 1991; Rounsley et al., 1995; Alvarez-Buylla et al., 2000; Liljegren et al., 2000; Pinyopich et al., 2003). These genes, which likely arose through gene duplication, have retained probable ancestral roles in reproductive development, including redundant roles in carpel identity, and have gained other roles in the current derived carpel structure. For example, *SHP1* and *SHP2*

have redundant functions in the promotion of valve margin specification (Liljegren et al., 2000) and can substitute for AG function in carpel specification (Pinyopich et al., 2003). All four *AG*-related genes (*AG*, *SHP1*, *SHP2*, and *STK*) have overlapping expression patterns in the placenta and developing ovule primordia (Rounsley et al., 1995; Ferrándiz et al., 1999). Although the double mutant *shp1 shp2* does not exhibit altered ovule development, the *stk* mutant does have ovule defects, but only in funiculus differentiation, in which each funiculus has more cells than the wild type and fails to develop an abscission zone (Pinyopich et al., 2003). By contrast, when the functions of all three genes are lost in the triple mutant *stk shp1 shp2*, fewer ovules are initiated and ovule development is severely disrupted (Pinyopich et al., 2003). Examination of the triple mutant phenotype seen in Figure 3D shows that the funiculus still is present, with the features expected for the *stk* mutant, whereas the distal portions of the ovules show conversion to carpel-like structures. This dramatic phenotype suggests that these genes are major identity determinants of at least the distal ovule structures.

The triple mutant phenotype has similarities with the *bel1* phenotype, because carpel structures can form from the region that produces integuments in both mutants. The phenotype of the triple mutant at early ovule stages has not been reported, so the timing of the manifestation of the phenotype is unknown. The loss of *STK*, *SHP1*, and *SHP2* function produces a more consistent homeotic change than that seen in *bel1* mutants, in that 95% of ovules show this conversion of the integuments compared with <15% in *bel1* (Ray et al., 1994; Pinyopich et al., 2003). If carpel identity is suppressed by the presence of integument identity, the triple mutant phenotype shows that *BEL1* cannot provide this identity without one of the three redundant genes, *STK*, *SHP1*, or *SHP2*. This could mean that *BEL1* is not active in a *stk shp1 shp2* background and may even be genetically downstream of these genes. An alternative model would be that *BEL1* is upstream of *STK*, *SHP1*, and *SHP2* and that the integument identity seen occasionally in the *ap2 ag bel1* mutant is provided by other unknown genes. Examination of quadruple mutants of *bel1* with *stk shp1* and *shp2*, as well as observation of the change in expression pattern of *bel1* in the triple mutant, will help to discriminate between these models.

In summary, *BEL1* and the *AG* clade all contribute to the identity of ovule structures. Without the identity provided by *STK*, *SHP1*, *SHP2*, and *BEL1*, the integuments acquire carpel features. Because the primary floral C class gene *AG* is expressed in the region, the primordia may revert to an underlying pathway of carpel development when the ovule identity factors are absent. No mutants or mutant combinations have been observed that result in the loss of the defining ovule features, funiculus, integuments, and nucellus, indicating that factors important for ovule identity remain to be discovered.

The importance of the *AG* clade in ovule development also has been demonstrated in other species. The *Petunia hybrida* genes *FLORAL BINDING PROTEIN7* (*FBP7*) and *FBP11*, which are 90% identical at the amino acid level, fall within the petunia *AG* clade (Angenent et al., 1995). Phylogenetic analyses indicate that *FBP7* and *FBP11* share the greatest similarity with *STK* (Theissen et al., 1996, 2000; Immink et al., 2003). The two petunia genes are first expressed in the placenta before ovule primordia emerge. There is some expression in young primordia, and expression increases in the funiculus and integuments (Angenent et al., 1995; Cheng et al., 2000). This expression pattern is similar to that of *STK*, *SHP1*, and *SHP2*. *FBP11* and *FBP7* have been reported to cause occasional formation of ovules on sepals, petals, and carpels when expressed ectopically (Colombo et al., 1995; Cheng et al., 2000). However, both the sepals and petals in these plants showed other phenotypes correlated with the acquisition of carpel identity. These ovules could be an indirect effect of the expression of *AG* homologs that promote carpel development. Cosuppression experiments with *FBP7* and *FBP11* showed a partial loss in ovule identity in a subset (7.5%) of transformants. In these plants, many ovules were converted to carpel- or style-like structures. Because the funiculi of petunia ovules are very short (Angenent et al., 1995), it is not possible to determine from the available data whether the integument or an entire primordium becomes carpelloid. The presence of some normal ovules even in those plants with undetectable FBP7 and FBP11 mRNA levels shows that ovule identity still was present. This finding indicates that cosuppression was incomplete or that other genes are capable of providing ovule identity.

In Arabidopsis, interactions between SEPALLATA (SEP) MADS box proteins and floral B and C function MADS box proteins, such as AP3 and AG, are necessary for the determination of floral organ identity (Pelaz et al., 2000, 2001a; Honma and Goto, 2001). Recent evidence suggests that the SEP proteins could be required for the determination of ovule identity in a manner similar to that of floral organ identity. The Arabidopsis gene *SEP3* (*AGL9*) is expressed in placentas and ovule primordia until embryogenesis (Mandel and Yanofsky, 1998). *SEP1* and *SEP2* are expressed in ovules beginning at floral stage 10, the time of integument emergence (Ma et al., 1991). Favaro et al. (2003) recently showed that *SEP1*/*sep1 sep2 sep3* mutant ovules have tissue transformations resembling those in *stk shp1 shp2* ovules. In addition, STK, AG, SHP1, and SHP2 were shown to interact with SEP proteins to form multimeric complexes (Favaro et al., 2003). Together, these results make a strong case for the importance of large MADS complexes in ovule identity. Recent reports in other species further support this hypothesis. In petunia, FBP7 and FBP11 (apparent STK orthologs) interact in vitro with FBP2 and FBP5, which are putative orthologs to the SEP proteins of Arabidopsis (Pelaz et al., 2000; Immink et al., 2002, 2003; Ferrario et al., 2003). In addition, the rice STK homolog OsMADS13 also interacts with SEP-related proteins from rice: OsMADS24 and OsMADS45 (Lopez-Dee et al., 1999; Favaro et al., 2002). These results from Arabidopsis, rice, and petunia support the model that the SEP proteins form complexes with STK, SHP1, SHP2, and possibly AG to maintain ovule identity in divergent species.

Certain Arabidopsis *ap2* alleles exhibit some conversion of ovules to carpelloid structures in the gynoecium (Modrusan et al., 1994), suggesting that *AP2* may suppress *AG* in ovules as well as in the outer whorls of the flower. Interestingly, double mutants of the *ap2*-like genes *LIPLESS* (*LIP1*) and *LIP2* in *Antirrhinum majus* show a similar phenotype (Keck et al., 2003). However, this carpelloidy is not caused by an increase in *PLENA* expression (the *AG* ortholog) in ovules, suggesting a different mechanism for C function regulation in *A. majus*.

PRIMORDIUM OUTGROWTH

The initiation of ovule primordia is not disrupted significantly by defects in ovule identity genes, as illustrated by the relatively normal appearance of the primordia that later form the carpelloid and undifferentiated mutant ovules discussed above. The recently described pattern of *CUC3* expression, which is observed in a ring surrounding the base of each primordium (Vroemen et al., 2003), indicates that the *CUC* genes may play a role in the location of primordium initiation. Outgrowth of the ovule primordium requires an unknown signal to direct cells in the placenta to expand and divide out of the plane of the developing septum. These processes appear dependent on metabolic energy to fuel growth and cell divisions. Mutations that show effects on the outgrowth of ovule primordia provide an interesting view of these basal requirements for organ outgrowth. An apparent requirement for metabolic energy in primordium outgrowth and cellular health is shown by the *huellenlos* (*hll*) mutant (Schneitz et al., 1998), which has lost a vital mitochondrial ribosomal protein (Skinner et al., 2001). The mutant is thought to have impaired mitochondrial function in ovule primordia, and the phenotype is short, degenerating primordia. Because of redundancy with the *HUELLENLOS PARALOG* (*HLP*) gene, the mitochondrial defect is manifest primarily in the gynoecia and ovules. Presumably, the cells of the primordia cannot perform normal cellular function and thus fail to undergo division and subsequently die. Interestingly, this loss of mitochondrial function reveals the action of other genes in promoting primordium growth. *ant* mutants have normal primordia that lack integuments. However, the *ant hll* double mutant has shortened primordia that lack obvious funiculi (Schneitz et al., 1998). The *hll* mutant also shows synergistic interactions with the *short integuments2* (*sin2*) mutant (Broadhvest et al., 2000). The double mutant ovule primordia are reduced severely to small outgrowths of only a few cells. Thus, ANT is clearly active in promoting cell proliferation here, as it is in shoot and floral meristems. This finding also sheds light on the possible role of the SIN2 protein, which appears to be involved in aspects of cell division in a way that is particularly sensitive to the reduction in metabolic health.

PATTERNING THE OVULE PRIMORDIUM

After the emergence of an ovule primordium, positional information along the proximal-distal axis must be interpreted so that the different regions of the primordium take on their characteristic fates. Three zones of differentiation are easily recognized: the distal zone becomes the nucellus, which gives rise to the megaspores and embryo sac; the central region is referred to as the chalaza and is the location of integument formation; and the proximal zone becomes the funiculus, a supporting stalk. There also are differences along the sides of the ovule facing the apical (stigma) and basal (receptacle) ends of the carpel. We use the terms ''gynoapical'' and ''gynobasal'' to represent these two directions along this axis, which is perpendicular to the proximal distal axis of the ovule. We use these terms in place of earlier terms such as anterior/posterior, dorsal/ventral, and abaxial/adaxial that have been used to refer to this axis (abaxial/adaxial will be reserved to designate the two faces of lateral organs). Differential growth of the funiculus and outer integument along the gynoapical/gynobasal axis results in the final S shape of the Arabidopsis ovule (Figure 1C). The mechanisms required for the establishment of these domains are largely unclear. However, we are beginning to identify components of the pathways that define the position and extent of integument growth along the proximal/distal and gynoapical/gynobasal axes.

Two genes are essential for integument initiation and also have important roles in proximal/distal patterning. The AP2 domain transcription factor ANT appears to have a role in promoting primordium outgrowth at a number of steps in reproductive development (Elliott et al., 1996; Krizek et al., 2000) and is required for integument initiation and growth (Elliott et al., 1996; Klucher et al., 1996; Baker et al., 1997). Severe *ant* alleles, such as *ant-4* and *ant-5*, lead to a complete absence of integuments, with the resulting ovules having only a rudimentary bulge in the chalazal region as a result of cell expansion (Figure 4D) (Baker et al., 1997). *ANT* is expressed throughout the young ovule primordia. However, by early stage 2, before integument initiation, its expression becomes restricted to the distal funiculus and chalaza (Elliott et al., 1996; Balasubramanian and Schneitz, 2000). Once integument cell divisions have begun, *ANT* expression becomes limited to the integument primordia and distal funiculus.

The *ant* mutant ovules are similar in appearance to those produced by *wuschel* (*wus*) mutants when the earlier effects of *wus* are mitigated (Figures 4D and 4E) (Gross-Hardt et al., 2002). The WUS homeodomain protein is required to maintain the shoot meristem, and *wus* mutants normally lack flowers. *WUS* expression was detected in the nucellar region of the ovule primordium (Gross-Hardt et al., 2002). Therefore, to determine the role of *WUS* during ovule development, Gross-Hardt et al. (2002) created *wus* mutants carrying a *CLAVATA1* promoter:*WUS* cDNA fusion transgene (*P-CLV1*:*WUS*). Because *P-CLV1* produces expression in meristems and not ovules, these plants were able to overcome the *wus* meristem and floral defects to reveal ovule effects of the mutation. The flowers of these plants contained ovules completely lacking integuments (Figure 4E).

The position of integument initiation along the proximal-distal axis of the ovule primordia is influenced by the novel nuclear protein NOZZLE/SPOROCYTELESS (NZZ/SPL) (Figures 4A and 4B) (Schiefthaler et al., 1999; Yang et al., 1999; Balasubramanian and Schneitz, 2000). One effect of *nzz/spl* mutations is to reduce the length of the nucellar region of the primordium. This could result from reduced nucellar growth attributable to a failure to produce a megasporocyte, consistent with a similar effect of this mutation on anther and pollen development (Yang et al., 1999). Alternatively, it could represent a direct distal shift in the position of integument formation along the proximal-distal axis, consistent with the observed increased length of the funiculus (Figures 4B and 4C) (Schiefthaler et al., 1999; Balasubramanian and Schneitz, 2000). This change in the location of integument initiation is associated with a distal expansion of the *ANT* expression domain (Balasubramanian and Schneitz, 2000). The ectopic *ANT* expression appears to be required for the *nzz* phenotype. *nzz ant* double mutants are *ant*-like, with no obvious reduction in nucellar tissue, indicating that a major role of NZZ is

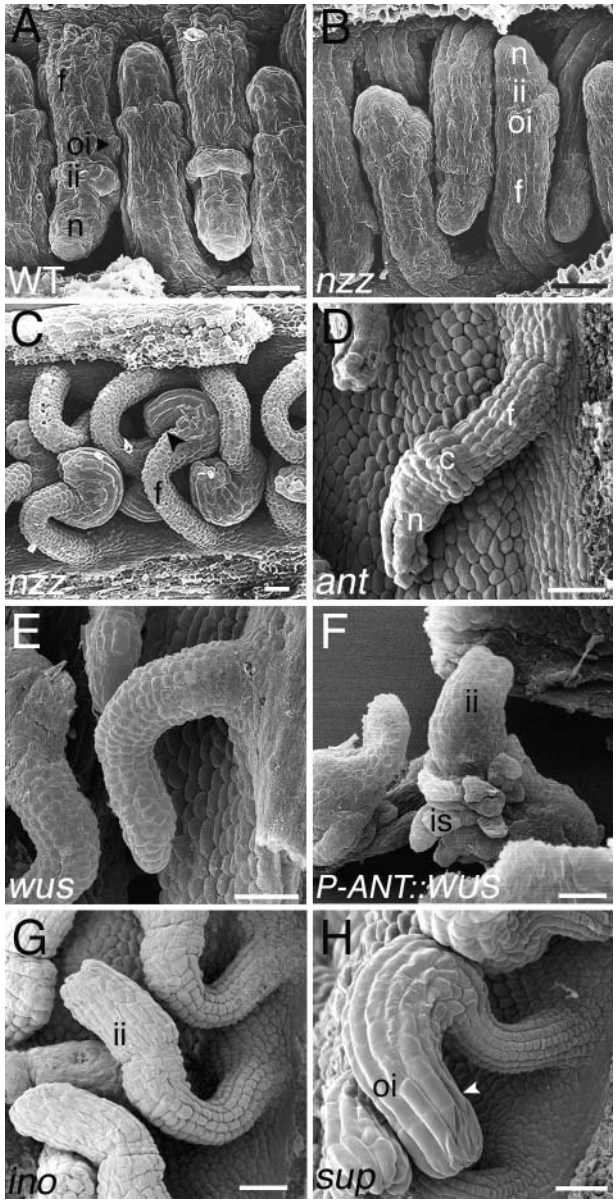

Figure 4. Mutations That Affect Ovule Integument Initiation.

(A) and **(B)** Wild-type (WT; **[A]**) and *nzz-2* **(B)** ovules just after integument initiation, at ovule stage 2-I. The *nzz* inner and outer integuments initiate more distally along the ovule primordia than do the wild-type integuments, resulting in *nzz* ovules with a shorter nucellus and a longer funiculus.

(C) to **(H)** Ovules at anthesis from *nzz-2* **(C)**, *ant-4* **(D)**, *wus-1* (with vegetative and floral effects complemented by a *P-CLV*:*WUS* transgene) **(E)**, *P-ANT*:*WUS* **(F)**, *ino-1* **(G)**, and *sup-5* **(H)** mutants. The distal shift in the integuments of *nzz* mutants results in an extended funiculus, as shown in **(C)**. In addition, ectopic growth of the gynoapical part of the outer integument results in incomplete fusion to the funiculus (arrowhead). *ant* ovules fail to produce integuments, but limited expansion of the cells of the chalaza produces the ridge shown in **(D)**. The typical *wus* mutant ovule, shown in **(E)**, displays no external sign of integument growth. Using the *ANT* promoter, ectopic expression of *WUS* in the chalaza and distal funiculus results in the production of multiple integument-like structures, as seen in **(F)**. Failure to form an outer integument leaves the inner integument exposed in *ino* mutants **(G)**. The ovule in **(H)** is from a *sup* mutant showing the ectopic growth of the outer integument on the gynoapical side of the ovule (arrowhead). c, chalaza; f, funiculus; ii, inner integument; is, integument structures; n, nucellus; oi, outer integument.

Bars = 25 μm. Images in **(A)** to **(C)** courtesy of S. Balasubramanian and K. Schneitz; **(E)** and **(F)** courtesy of R. Gross-Hardt and T. Laux; **(H)** courtesy of R.J. Meister.

the spatial limitation of *ANT*. Therefore, it appears that the chalazal region is defined in part by the distal extent of *ANT* expression at stage 2-I and that *NZZ* influences this through negative regulation of *ANT* in the nucellus. This implies a close link between the pattern of *ANT* expression and the location of integument initiation. Later in ovule development, *nzz* mutants show an overproliferation of the nucellus and the gynoapical region of the outer integument (Figure 4C). Because *NZZ*/*SPL* has been reported to be expressed throughout the ovule primordium (Balasubramanian and Schneitz, 2000) or only in the nucellar region (Yang et al., 1999), the mechanisms by which *NZZ*/*SPL* regulates the *ANT* expression domain are unclear.

The link between *ANT* expression and chalazal positioning can be tested by altering the expression of *ANT*. Unfortunately, the effects on ovule development of ectopic *ANT* expression from transgenes have been difficult to interpret. The 35S promoter of *Cauliflower mosaic virus* (35S) is used commonly in transgenic overexpression/ectopic expression studies. However, the activity of this promoter is very low in the ovule primordia at inception and increases to a higher, but still variable, level throughout the ovule at later stages (Jenik and Irish, 2000). *35S*:*ANT* transgenes result in variable ovule phenotypes with reduction of inner integument growth, overproliferation of the nucellus, and ectopic growth of the outer integument on the gynoapical side of the ovule (Krizek, 1999). In this case, the relatively stronger endogenous *ANT* expression may provide the location for inner integument initiation, with this boundary somewhat blurred by the weaker but more widespread expression from the 35S promoter. This could lead to a less efficient initiation followed by retarded growth. Alternatively, expression of *ANT* in tissues underlying the inner integument may be inhibitory to its growth. The phenotypic variability observed in the *35S*:*ANT* ovules also is likely to be attributable to the weak and variable activity of the 35S promoter. The late overproliferation of the nucellus and gynoapical outer integument is similar to that in *nzz* mutants, which also have ectopic *ANT* expression in the nucellus (Krizek, 1999; Balasubramanian and Schneitz, 2000).

An appropriate pattern of *WUS* expression also is important to define the region of an ovule primordium capable of giving rise to integuments. Surprisingly, the *WUS* expression pattern does not appear to include the cells from which the integuments derive. Even though the effects of the *wus* mutation on ovule development (the absence of integuments; Figure 4E) appear specific to the chalazal region, WUS is restricted to the nucellus (Gross-Hardt et al., 2002). This fact suggests that *WUS* promotes integument growth through a non-cell-autonomous mechanism.

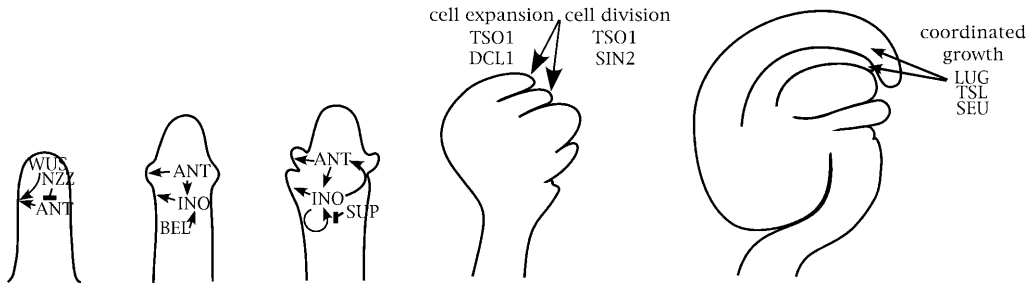

Figure 5. Summary Models for Gene Interactions in Ovule Development.

Before integument initiation, *WUS* and *ANT*, which are expressed in close proximity, induce integument formation at the distal boundary of *ANT* expression, whereas *NZZ* determines the distal extent of *ANT* expression. Just before integument initiation *INO* expression is activated on the gynobasal side of the ovule at the base of the region that will give rise to the outer integument. This expression is dependent on *ANT* and *BEL1* activity. Once both the inner and outer integuments have emerged, *INO* is expressed in the abaxial cells of the outer integument. ANT and INO are required to maintain *INO* expression in these cells, and INO supports *ANT* expression. The INO autoregulatory loop is negatively regulated by SUP, which is required to restrict INO expression to the gynobasal side of the ovule. *SIN2* and *TSO1* promote integument cell proliferation, whereas *TSO1* and *DCL1* regulate integument cell expansion. *LUG*, *SEU*, and *TSL* are required for the coordinated growth of the inner and outer integuments, ensuring that the outer integument supersedes and encloses the inner integument of an ovule at anthesis.

WUS is expressed normally in the nucellar region of the ovule primordia from very early stages, with the highest level of expression at stage 2-II to 2-III, coincident with integument initiation (Gross-Hardt et al., 2002). The importance of this expression pattern is demonstrated by the phenotype of plants ectopically expressing *WUS* in the chalazal region under the control of the *ANT* promoter. The *P-ANT*:*WUS* plants produce what appear to be ectopic integuments that form reiteratively toward the base of the ovule (Figure 4F). The first integuments produced by these ovules appear similar to those in the wild type, which demonstrates that *WUS* does not need to be excluded from the cells giving rise to the inner integument. However, the ectopic integuments that do form are highly irregular. These irregular outgrowths appear to have some integument identity, because they exhibit *ANT* expression in an integument-like pattern. However, whether they have outer or inner integument characteristics is undetermined. The *P-ANT*:*WUS* plants indicate that WUS is capable of inducing ectopic integument growth in the context of the ovule.

Why are multiple integument-like structures formed in *P-ANT*:*WUS* plants? This is likely attributable to *P-ANT*:*WUS* expression in the distal funiculus and chalaza. This region then would include multiple new boundaries of *ANT* and *WUS* activity, created by the upregulation of endogenous *WUS* by *P-ANT*:*WUS* in the existing central chalazal region and the downregulation of *ANT* in this central chalazal region upon integument outgrowth (Gross-Hardt et al., 2002) (Figure 5). *ANT* expression is maintained in the distal funiculus, whereas *WUS* is expressed in the distal funiculus and the chalaza. Therefore, a new expression boundary is created at the junction of the distal funiculus and the chalaza. In wild-type plants, when *ANT* is downregulated in the chalazal region, the domains of *ANT* and *WUS* expression are not in close proximity. Because only two integuments are formed in wild-type plants, it is likely that ANT needs to be within a certain distance of WUS to induce integument initiation. These data are consistent with a model whereby, in wild-type plants, the spatial relationship between *WUS* expression in the nucellus and *ANT* expression in the chalaza determines the site of inner integument growth and *NZZ* is required to establish the distal extent of *ANT* expression (Figure 5). The mechanisms that establish the *WUS* expression domain also are unknown.

OUTER INTEGUMENT INITIATION AND ORIENTATION

Although ANT and WUS are essential for the formation of both integuments, outgrowth of the outer integument also requires the activity of a third transcription factor, the YABBY protein INNER NO OUTER (INO) (Baker et al., 1997). Strong *ino* mutants completely lack an outer integument but otherwise are phenotypically wild type (Figure 4G) (Gaiser et al., 1995; Baker et al., 1997; Schneitz et al., 1997). Therefore, *INO* is required specifically for outer integument formation (Bowman and Smyth, 1999; Villanueva et al., 1999).

INO expression is first observed at stage 2-I just before the initiation of either integument. Expression is only on the gynobasal side of the ovule, in the proximal half of the region of cells that will give rise to the outer integument (Figure 1E) (Villanueva et al., 1999; Meister et al., 2002). The sequential initiation of integument growth, with the inner integument emerging first, may indicate that the initiation of outer integument growth, and hence the position of the initial expression of *INO*, may be dependent on the position of the inner integument. This notion is supported by the fact that the absence of the inner integument is always accompanied by the absence of the outer integument in known mutants. Because the position of the inner integument is linked closely to the location of *ANT* expression, the model described above implies a link between *ANT*, *INO*, and the location of the outer integument. Experimental results support this link. In the *nzz* mutant, both *INO* expression and the site of initiation of

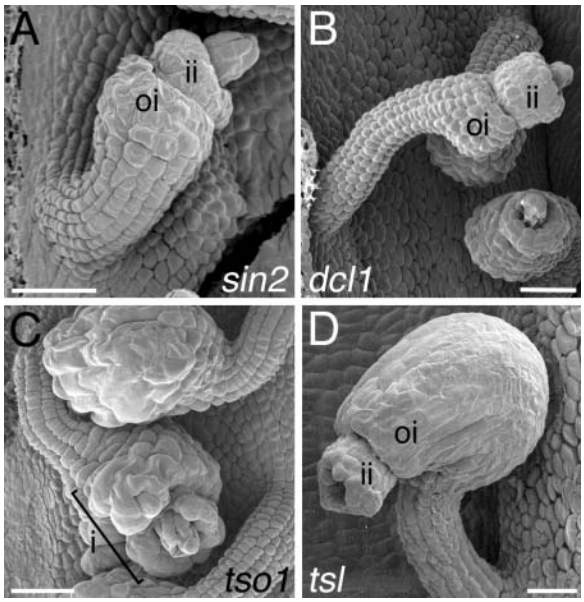

Figure 6. Ovules of Integument Cell Proliferation and Expansion Mutants at Anthesis.

(A) *sin2-1* integument cells stop dividing prematurely.
(B) *dcl1-7* mutants have normal numbers of cells in the integuments, but the cells fail to expand.
(C) *tso1-3* integument cells undergo aberrant cell expansion and cell division.
(D) *tsl-1* mutants have reduced proliferation of the outer integument and an overproliferation of the inner integument, resulting in its protrusion beyond the outer integument.
i, integument; ii, inner integument; oi, outer integument. Bars = 20 μm. Images in **(B)** and **(C)** courtesy of J. Broadhvest; **(D)** courtesy of J.L. Roe and P.C. Zambryski.

the outer integument are closer to the distal end of the ovule primordium than in the wild type, as would be expected from the shift in the pattern of *ANT* expression (Figures 4A and 4B) (Balasubramanian and Schneitz, 2000). In addition, in strong *ant* mutants, such as *ant-72F5* and *ant-5*, *INO* expression is delayed and at a lower level than in the wild type (Balasubramanian and Schneitz, 2000; Meister et al., 2004) and may be absent in *ant-4* (Meister et al., 2004). Thus, ANT may promote outer integument development by facilitating inner integument development and through positive regulation of *INO* (Figure 5).

The interaction of *ANT* with *INO* may be bidirectional. In the strong *ino-2* mutant, *ANT* expression is at normal levels in the inner integument but is reduced in the region that normally would produce the outer integument (Balasubramanian and Schneitz, 2002). This implies that *INO* positively affects *ANT* expression. Thus, *ANT* and *INO* may constitute an autoregulatory loop in which each reinforces the expression of the other (Figure 5). *INO* has been shown to positively affect its own expression; both *INO* transcript accumulation and a *P-INO:GUS* reporter gene showed reduced expression in an *ino* mutant background (Villanueva et al., 1999; Meister et al., 2002). The autoregulation of *INO* may be direct, or the autoactivation of *INO* could be a secondary effect of the *INO* activation of *ANT* expression. Transgenic studies show that regulation by both factors is transcriptional because it is mediated by the *INO* promoter sequence (Meister et al., 2004). The positive regulation of *ANT* by *INO* may provide an explanation for the restoration of the normal length of the nucellar region in *ino nzz* double mutants relative to *nzz* single mutants (Balasubramanian and Schneitz, 2002). The distal shift of *ANT* expression hypothesized to contribute to the reduction in the size of the nucellar region may not occur in the absence of the positive influence of *INO* on *ANT* expression.

The expression of *INO* in *ant-72F5* ovules suggests that factors in addition to ANT must contribute to the *INO* expression pattern. A likely candidate is the homeodomain protein BEL1 (Reiser et al., 1995). *BEL1* is expressed in the chalazal region from stage 1, independent of *ANT* function. Mutations in this gene result in delayed or reduced *INO* expression (Balasubramanian and Schneitz, 2000; Meister et al., 2004). Because both *BEL1* and *ANT* expression domains occur throughout the chalazal region, additional factors must be required to define the more limited *INO* expression pattern. Our model suggests that at least some of these factors would be associated with the induction of the inner integument.

INO expression is regulated not only along the proximal-distal axis but also along the gynoapical-gynobasal axis. *INO* expression initiates only toward the gynobasal side of the ovule, where the outer integument initiates and exhibits greatest growth (Figures 1E to 1G). Throughout ovule development, this pattern of expression is maintained (Villanueva et al., 1999). The maintenance of the expression pattern is dependent on the putative zinc finger transcription factor SUPERMAN (SUP) (Meister et al., 2002). By stage 2-V, *sup-5* mutants exhibit *INO* expression on both the gynobasal and gynoapical sides of the ovule primordium, resulting in ectopic growth of the outer integument on the gynoapical side of the ovule. The SUP-mediated negative regulation requires INO function, because replacement of the *INO* coding sequence with that of the paralogous *CRABS CLAW* gene also results in ectopic growth and *INO* promoter activity on the gynoapical side of the ovule (Meister et al., 2002). The simplest model to explain the involvement of INO function in the negative regulation by *SUP* is for SUP to interfere with the *INO* positive autoregulation (Figure 5). Although the interplay between positive regulation by INO and negative regulation by SUP can explain the maintenance of the asymmetrical pattern of *INO* expression, the factors for the initial induction of this pattern remain unknown. The overproliferation of the gynoapical outer integument of *nzz* mutants is similar to the *sup* ovule phenotype (Balasubramanian and Schneitz, 2000). As seen in *sup* mutants, the gynoapical overproliferation is accompanied by the extension of *INO* expression to the gynoapical side of the ovule (Balasubramanian and Schneitz, 2000, 2002). This suggests that *NZZ* is important for the negative regulation of *INO* by SUP. This effect may be indirect. A distal shift in the chalazal domain without an accompanying shift in SUP activity would result in *INO* expression distal to the range of SUP activity, and the influence of SUP would be reduced. This would allow a gynoapical expansion of the INO expression domain in *nzz* mutants.

Integument Extension

Unlike leaf morphogenesis, integument development appears to be relatively sensitive to alterations in cell division and cell expansion. This is reflected in the number of mutants that affect the size of the integuments without altering orientation or identity (Robinson-Beers et al., 1992; Baker et al., 1997; Schneitz et al., 1997). The propensity for mutational alteration may be attributable to the simplicity of these structures, having a relatively small number of cells and therefore less capacity to adapt to growth alterations. There also is a limited period during which the integuments can develop, because lack of fertilization results in degeneration of the ovule. In addition, mutations are easily identified, because a minor disruption of integument morphogenesis can lead to reduced female fertility. Below, we discuss the better characterized genes involved in integument morphogenesis.

An apparent difference in the mechanism of growth between the two integuments is highlighted by a lack of only the outer integument in the *ino* mutants (Baker et al., 1997). This and the expression of *INO* in the outer layer of the outer integument suggests that *INO* not only participates in establishing the polarity of the ovule primordium but also appears to provide abaxial identity in the outer integument (Villanueva et al., 1999; Meister et al., 2002). The requirement of *INO* for outer integument growth suggests that an organ boundary resulting from the juxtaposition of adaxial and abaxial domains, such as that required for leaf blade outgrowth, is required for outer integument growth (Waites and Hudson, 1995; Siegfried et al., 1999). The ovules of the less severe *ino-4* mutant demonstrate that *INO* is not only required for the initial outgrowth of the outer integument but also for the sustained growth of this structure (Villanueva et al., 1999). A similar dependence on abaxial/adaxial factors for inner integument growth has not been observed. In addition, none of the YABBYs, which are required for the polarity and growth of other plant organs, have been found to be expressed in the inner integument. This finding underscores a fundamental difference in the determination of polarity and growth between outer and inner integuments consistent with their separate evolutionary derivation (Stebbins, 1974; Stewart, 1983). Understanding inner integument growth may reveal additional mechanisms for plant organ growth.

During wild-type integument morphogenesis, there is coordination between inner and outer integument growth (Robinson-Beers et al., 1992). As mentioned above, outer integument initiation closely follows the initiation of the inner integument and may even be dependent on the inner integument. Subsequently, the two integuments grow in a concerted manner over the nucellus. Several mutations disrupt the coordination of growth between the inner and outer integuments. Three examples are the *lug*, *tsl*, and *seuss* (*seu*) mutants, which all have similar effects on integument growth (Roe et al., 1997b; Franks et al., 2002). The *lug*, *seu*, and *tsl* mutants appear to have delayed or reduced outer integument growth and a subsequent overproliferation of the inner integument, resulting in a protruding inner integument at anthesis (Figure 6D). TSL is a nuclear Ser/Thr protein kinase and therefore is a good candidate for communicating signals between the developing inner and outer integuments (Roe et al., 1997a). LUG and SEU, which are thought to act together as transcriptional corepressors, may respond to such signals and negatively regulate genes that promote inner integument growth (Franks et al., 2002). Consistent with LUG and TSL acting in the same pathway, the *lug tsl* double mutant has a protrusion of the inner integument similar to that of either single mutant (Roe et al., 1997b). It also is possible that the enhanced growth of the inner integument is not a primary effect of the mutations but rather is an indirect effect of delayed or slowed outer integument growth. It is notable that the partial loss-of-function *ino-4* mutant results in the production of a short outer integument (Villanueva et al., 1999). Although inner integument growth initially is normal, by stage 14, the inner integuments are abnormally elongated (Villanueva et al., 1999). This abnormal extension might result from a simple loss of mechanical confinement, attributable to the failure of the outer integument to enclose the inner integument, or from differential resource availability resulting from less resource utilization by the stunted outer integument.

Several additional genes have been found to influence the growth of both inner and outer integuments, apparently participating in different pathways that regulate cell division and/or cell expansion. *SIN2* is required to sustain cell divisions in the developing integuments (Figure 6A) (Broadhvest et al., 2000). By contrast, the integuments of *dicer-like1* (*dcl1*, formerly *short integuments1*) mutants are shortened as a result of reduced cell elongation along the proximal-distal axis of the ovule (Figure 6B) (Robinson-Beers et al., 1992; Ray et al., 1996a; Schauer et al., 2002). The DCL1 RNase III/RNA helicase is thought to be involved in the production or activity of small RNAs associated with the translational regulation of developmental genes (Jacobsen et al., 1999; Golden et al., 2002). Severe malformation of the inner and outer integuments results from mutations in *TSO1* (Figure 6C) (Hauser et al., 1998). The novel nuclear TSO1 protein is required for appropriate directional cell expansion and cell plate formation (Liu et al., 1997; Hauser et al., 2000). All of the double mutant combinations between *sin2*, *tso1*, and *dcl1* result in additive phenotypes (Hauser et al., 1998; Broadhvest et al., 2000), suggesting that these genes all act in separate pathways to regulate cell division and/or cell expansion during integument development.

During leaf development, cell division and cell expansion can be somewhat plastic—that is, cell division is capable of compensating for alterations in cell expansion, or conversely, cell expansion can compensate for alterations in cell division, to maintain normal leaf morphology (Tsukaya, 2003). However, there are cases in which this does not occur, leading to the proposal that plants have both cell division–dependent and cell division–independent mechanisms of morphogenesis (Fleming, 2002). In integument growth, effects on both cell division (as in *lug*, *seu*, *tsl*, and *sin2*) and cell expansion (as in *dcl1*) can independently affect integument size, indicating a lack of compensatory mechanisms. These mutations also have pleiotropic developmental effects, particularly affecting flower development (Roe et al., 1993, 1997b; Liu and Meyerowitz, 1995; Ray et al., 1996a, 1996b; Liu et al., 1997; Hauser et al., 1998; Jacobsen et al., 1999; Broadhvest et al., 2000; Franks et al.,

2002; Golden et al., 2002). This suggests that these integument growth regulators are capable of responding to a variety of developmental cues, with their function determined by the tissue context in which they act.

PERSPECTIVE

The relative structural simplicity of the ovule belies the complexity of the underlying regulation necessary for the formation of this structure. Genes now identified as essential for normal ovule morphogenesis encode a wide variety of transcription factors, putative corepressors, a protein kinase, a mitochondrial ribosomal subunit, and a component of the RNA interference pathway. The activities of these proteins demonstrate the importance of gene regulation, signal detection and transduction, and cellular metabolism in ovule morphogenesis, likely reflecting the complexity of plant development in general. Advances have been made in understanding some interactions of genes involved in later stages of ovule patterning and integument formation. Transcriptional regulation and polarity determination appear to play key roles in these processes. The fact that links between transcriptional regulation, signal transduction, and metabolism have not yet been elucidated indicates that many additional players in ovule morphogenesis remain to be identified.

Several features of ovule initiation and morphogenesis show interesting parallels with other aspects of plant development. For example, in situ data show a juxtaposition of the expression of adaxial (*REV* and related genes) and meristem (*STM*) factors at the placenta, where ovule primordia form. Such a juxtaposition has been associated with axillary meristem formation (McConnell and Barton, 1998), suggesting the possibility of related mechanisms for the formation of secondary shoots and ovules. Integument initiation also has parallels with leaf initiation. Leaves initiate in regions of *ANT* expression from which the expression of the meristem maintenance gene *STM* has been excluded (Long and Barton, 1998). Integuments emerge from a region of *ANT* expression from which the expression of the meristem maintenance gene *WUS* has been excluded. Outer integument growth appears to require abaxial expression of the YABBY gene *INO*. Abaxial YABBY gene expression is a feature common to all other lateral organs of Arabidopsis, including leaves, sepals, petals, stamens, and carpels. Together, these common features support the idea that an ovule is a shoot-like structure (caulome) and that the outer integument may have evolved from a leaf or leaf-derived structure (phyllome). However, consistent with ovules as specialized reproductive shoots, the positioning of ovule regions is affected by *NZZ*/*SPL*, a gene associated with sporogenic structures.

The well-known roles of MADS box proteins in floral organ identity parallel the overlapping roles of some genes in ovule identity. In floral organ identity, the A, B, and C class MADS box proteins (AP1, AP3 and PISTILLATA, and AG) are primary identity determinants that appear to require direct interaction with the SEP class of MADS proteins to manifest their identity functions (Honma and Goto, 2001; Pelaz et al., 2001b). MADS box proteins involved in ovule identity include AG and its closest relatives STK, SHP1, and SHP2. Initial evidence indicates that SEP proteins also may interact with the ovule identity factors and thus may be involved in ovule identity determination. However, ovule identity also is dependent on BEL1 activity, and no parallel function of a homeodomain protein has been found for floral organ determination.

We know relatively little about the genes that regulate the outgrowth of the ovule primordia. Other than HLL, ANT, and SIN2, each of which appears to play a role in this process, factors that regulate ovule outgrowth have yet to be identified. This is a key gap in our understanding of ovule development, because it is likely that these genes establish the expression patterns of the genes that are important for proximal-distal and gynoapical-gynobasal axis determination.

A complicating factor in the genetic dissection of ovule developmental pathways is that many mutations that affect ovule development may have pleiotropic effects on earlier stages of plant development that can mask their effects on ovules. A specialized vector for the mitigation of vegetative and floral effects of *wus* mutants was required before the role of *WUS* in ovule development was revealed (Gross-Hardt et al., 2002). Similar methods will be required to determine the ovule development roles of other meristem genes such as *STM*. Many other genes also may have unsuspected roles in ovule development that remain undetected. Emerging tools of transcriptional profiling and enhancer trap analysis can link genes to ovule development on the basis of expression pattern alone. The ongoing development of useful tools, such as placenta- and ovule-specific promoters, RNA interference technology, and libraries of insertional mutants, will facilitate reverse-genetic approaches to determine the function of candidate genes in ovule development.

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