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Roles of Polarity Determinants in Ovule Development

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Abstract

Summary

Ovules are the female reproductive structures that develop into seeds. Angiosperm ovules include one, or more commonly two, integuments that cover the nucellus and female gametophyte. Mutations in *Arabidopsis KANADI* (*KAN*) and *YABBY* polarity genes result in amorphous or arrested integument growth, suggesting that polarity determinants play key roles in ovule development. We show that the *Class III Homeodomain Leucine-Zipper* (*HD-ZIPIII*) genes *CORONA (CNA), PHABULOSA (PHB),* and *PHAVOLUTA (PHV)* are expressed adaxially in the inner integument during ovule development, independent of *ABERRANT TESTA SHAPE* (*ATS*, also known as *KANADI4*) activity. Loss of function of these genes leads to aberrant integument growth. Additionally, over-expression of *PHB* or *PHV* in ovules is not sufficient to repress *ATS* expression, and can produce phenotypes similar to those of the *HD-ZIPIII* loss of function lines. The absence of evidence of mutual negative regulation by KAN and HD-ZIPIII transcription factors is in contrast to known mechanisms in leaves. Loss of *HD-ZIPIII* activity can partially compensate for loss of *ATS* activity in the *ats cna phb phv* quadruple mutant, demonstrating that *CNA/PHB/PHV* act in concert with *ATS* to control integument morphogenesis. In a parallel pathway, *ATS* acts with *REVOLUTA (REV)* to restrict *INNER NO OUTER (INO)* expression and outer integument growth. Based on these expression and genetic studies we propose a model in which a balance between the relative levels of adaxial/abaxial activities, rather than the maintenance of boundaries of expression domains, is necessary to support laminar growth of the two integuments.

Keywords

integument; adaxial-abaxial polarity; HD-ZIPIII; KANADI; YABBY; *Arabidopsis*

Introduction

In flowering plants, ovules are critical female reproductive structures that develop into seeds. Angiosperm ovules include one, or more commonly two, integuments that cover the nucellus and female gametophyte. After fertilization the integuments become the seed coat. Ovule ontogeny has been well characterized in *Arabidopsis* (Robinson-Beers *et al.*, 1992;

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Schneitz *et al.*, 1995) and many genes involved in ovule development have been identified (Skinner *et al.*, 2004). Recent genetic studies have shown that mutations in putative polarity determinants, such as members of the YABBY and KANADI gene families, result in amorphous or arrested integument growth (Eshed *et al.*, 2001; McAbee *et al.*, 2006; Villanueva *et al.*, 1999), suggesting that such determinants play key roles in ovule development.

ABERRANT TESTA SHAPE (*ATS*, also referred to as *KANADI4* (*KAN4*)) is a member of the *KANADI* gene family that is necessary for laminar extension of the inner integument and for maintaining integument separation (McAbee *et al.*, 2006). In *ats* mutant ovules, the two integuments fail to originate as separate structures, resulting in a single 'fused' integument, and leading to aberrant heart-shaped seeds (Léon-Kloosterziel *et al.*, 1994; McAbee *et al.*, 2006). Loss of function of two other *KANADI* family members, *KAN1* and *KAN2*, results in an amorphous outer integument and a normal inner integument, implying that these polarity determinants are necessary for laminar extension of the outer integument (Eshed *et al.*, 2004; McAbee *et al.*, 2006). A dominant allele of one *Class III Homeodomain Leucine-Zipper* (*HD-ZIPIII*) gene, *PHABULOSA* (*PHB*), displays incomplete integument formation, implying that these transcription factors may also play important roles in ovule patterning and growth (McConnell and Barton, 1998).

Based on current knowledge regarding the expression and roles of polarity genes during leaf morphogenesis, a model has been proposed to explain initiation and extension of integuments and ovule mutant phenotypes such as *ats* (McAbee *et al.*, 2006). In leaves, the combined expression of *HD-ZIPIII* transcription factors provides adaxial identity, while *YABBY* and *KANADI* family members are abaxial determinants (Bowman *et al.*, 2002; Emery *et al.*, 2003; Kerstetter *et al.*, 2001; McConnell and Barton, 1998; McConnell *et al.*, 2001; Siegfried *et al.*, 1999). Loss of a polarity function can result in arrested and/or radialized lateral organs, leading to the hypothesis that the juxtaposition of adaxial and abaxial characters is required in order to properly define locations of laminar growth (Bowman *et al.*, 2002; Eshed *et al.*, 2001; Eshed *et al.*, 2004; Waites and Hudson, 1995; Waites *et al.*, 1998). McAbee et al. (2006) proposed that *ATS* acts as an abaxial determinant in the inner integument, while *KAN1* and *KAN2* act in conjunction with a *YABBY* gene (*INNER NO OUTER* (*INO*)) to specify abaxial cell fate in the outer integument. Furthermore, McAbee et al. (2006) hypothesized that *HD-ZIPIII* transcription factors act in both the outer and inner integuments as adaxial determinants.

While there is evidence to support the idea that abaxial determinants are required for initiation and maintenance of integument growth (Eshed *et al.*, 2001; McAbee *et al.*, 2006; Villanueva *et al.*, 1999), a role for adaxial determinants in ovules has not yet been demonstrated. We sought to further define the role of polarity establishment in integument growth through expression analyses, genetic and transgenic studies of HD-ZIPIII and ATS transcription factors.

In addition to their roles in leaves, we have found that *CORONA* (*CNA*), *PHB*, *PHAVOLUTA* (*PHV*) and *REVOLUTA* (*REV*) also regulate ovule development. We show these *HD-ZIPIIIs* act in concert with *ATS* to control patterning and laminar growth of both

the inner and outer integument. These results provide evidence that a polarity establishment pathway is required for integument formation, and also reveal differences between the pathways utilized in ovule and leaf development. In light of these new findings, we posit a model in which a balance between levels of polarity determinants acts to mediate integument development.

Results

The *HD-ZIPIII* genes *PHB*, *PHV*, and *CNA* are expressed in a polar fashion during ovule development

Based on previous genetic studies (Eshed *et al.*, 2001; McAbee *et al.*, 2006), we hypothesized that *HD-ZIPIII* genes may function as adaxial determinants during integument morphogenesis. There are five *HD-ZIPIII* genes in *Arabidopsis: CNA/ATHB15, PHB, PHV, REV*, and *ATHB8*. The patterns of *PHB* and *REV* have been previously examined during ovule development (Sieber *et al.*, 2004a). Herein we focus on expression of *PHV*, and *CNA*, and reexamine *PHB* as a control because of their demonstrated roles in leaf development, and an indication of a role in ovule development (McConnell and Barton, 1998; Sieber *et al.*, 2004a). *ATHB8* expression has been previously shown to be primarily associated with vascular development (Prigge *et al.*, 2005) and this gene was therefore not examined in this study. Expression patterns of *PHB*, *PHV*, and *CNA* were examined in wild-type ovules using *in situ* hybridization with gene-specific probes. In stage 2 ovules (stages according to (Schneitz *et al.*, 1995)) *PHB* mRNA was detected only in the inner integument, specifically in the cell layer adjacent to the nucellus (Figure 1a and Figure S1a,b). In later stages, hybridization was also seen in the vasculature of wild-type ovules (Figure 1a). This expression pattern is consistent with an earlier report (Sieber *et al.*, 2004a). *PHV* and *CNA* were expressed in similar patterns in ovules (Figure 1c and 1e, respectively; Figure S1e, f, i), with inner integument specific expression being present as early as stage 1 ovules (Figure 1e). Altogether the observed *PHB*, *PHV*, and *CNA in situ* hybridizations present an adaxial pattern in the inner integument (as defined in (McAbee *et al.*, 2006)).

During polarity establishment of leaves, *HD-ZIPIII* expression patterns are refined through a combination of microRNA regulation and repression by *KANADI* genes (Bowman *et al.*, 2002; Kidner and Timmermans, 2007). Given that *ATS* is the only *KANADI* gene known to be active in the inner integument, we wanted to test whether *ATS* is required to restrict the expression of *PHB*, *PHV*, and/or *CNA* during ovule development. In loss of function *ats* ovules we observed expression patterns for *PHB*, *PHV*, and *CNA* that did not differ from those observed in wild type (for *PHB* compare Figure 1b to 1a; for *PHV* compare Figure 1d to 1c; for *CNA* compare Figure 1f to 1e; see Figure S1 for additional comparisons). This suggests *ATS* activity is not required to delineate *PHB*, *PHV*, nor *CNA* expression patterns during ovule development.

PHB, *PHV*, and *CNA* are required for integument morphogenesis

In order to examine the collective role(s) of *PHB*, *PHV*, and *CNA* during ovule development we took a genetic approach. These genes overlap in expression patterns as well as function and therefore single and double mutants appear phenotypically wild type (Prigge *et al.*,

2005). A recent study of these genes described the *cna-2 phb-13 phv-11* triple mutant as having “short integuments” in addition to other developmental defects such as extra carpels (Prigge *et al.*, 2005).

In our examination of *cna-2 phb-13 phv-11* we found the ovule phenotype to be highly variable (Figure 2). In some cases, *cna-2 phb-13 phv-11* ovules initiated both integuments normally, resulting in nearly wild-type ovules (Figure 2a, e bottom right corner). More frequently, ovules exhibited a protruding inner integument and a superficially shorter outer integument at maturity (Figure 2b, f top right corner). This indicated a disruption in the coordination of timing of initiation and/or growth of the two integuments. Despite this morphogenetic disconnect between the two integuments, the outer integument still retained its anatropous form. We also observed ovules that phenocopied the ovules of *ino* mutants (Baker *et al.*, 1997; Villanueva *et al.*, 1999) (Figure 2c, e) having an inner integument and an absent or rudimentary outer integument. Ovules with an amorphous, globular structure in place of the integuments and an exposed nucellus were also commonly observed (Figure 2d–f). In general, it appears that the combined loss of *CNA*, *PHB* and *PHV* function leads to abnormal integument development. Surprisingly, this effect is not restricted to the inner integument, as would be predicted from their ovule expression patterns depicted in Figure 1.

Gain of function *phb-1d* mutants display ectopic *PHB* expression as a result of microRNA insensitivity (Reinhart *et al.*, 2002; Rhoades *et al.*, 2002). In *phb-1d* ovules the timing of integument initiation and growth is disrupted, producing ovules with elongated inner integuments (McConnell and Barton, 1998; McConnell *et al.*, 2001; Sieber *et al.*, 2004a). We examined *phv-1d* ovules to compare them to those of *phb-1d*. In *phv-1d* plants we observed three classes of ovules: *phb-1d*-like, *ino*-like and wild type (Figure 2h, i). Because these dominant mutations represent microRNA-resistant alleles of *PHB* and *PHV* (McConnell *et al.*, 2001), these data imply that proper regulation of *PHB* and *PHV* expression patterns via miR165/166 action is required for normal integument development. Additionally, these phenotypes were similar to those observed in the *cna phb phv* triple loss-of-function mutant suggesting that a relative level of adaxial activity produced by *PHB*, *PHV* and *CNA* may directly influence integument morphogenesis.

ATS and *HD-ZIPIII* functions are required to maintain integument development

We crossed *cna-2 phb-13 phv-11* with *ats-3 phb-6 phv-5 rev-9/+* in order to further examine the roles of *HD-ZIPIII* genes in the context of *ATS* function. We were able to identify *ats cna phb phv* and *ats cna phb phv rev/+* mutants in the F_2 population by PCR-based genotyping. While less than 10% of *ats cna phb phv* ovules had a wild type appearance (Figure 3d) no wild-type ovules were observed in *ats cna phb phv rev/+*. More frequently, *ats cna phb phv* ovules exhibited arrested outer integument growth (Figure 3e, f) and partial inner integument growth (Figure 3e, f; note the naked nucellus in 3f and the amorphous inner integument in 3e). Loss of one copy of *REV* in this mutant background enhances these defects, producing ovules with arrested inner and outer integument growth (Figure 3g, h). While both mutant combinations displayed a range of phenotypes, the *ats cna phb phv rev/+* mutants were more severely affected than the *ats cna phb phv* mutants (compare Figure 3g–h to d–f) suggesting that *REV* activity does contribute to integument growth.

Notably, the combined loss of *CNA*, *PHB*, and *PHV* function can suppress the integument fusion Ats$^-$ phenotype. In *ats* ovules the integuments are congenitally fused, resulting in a unitegmic ovule (McAbee *et al.*, 2006)(Figure 3a). However *ats cna phb phv* and *ats cna phb phv rev/+* ovules initiated separate inner and outer integuments (Figure 3c and e–h, respectively; compare Figure 3a to Figure 3c). It is worthwhile to note that this suppression is not observed in other mutant combinations tested, i.e. *ats phb phv rev/+* plants (see Figure 4), suggesting that loss of *CNA* activity (in the absence of *PHB* and *PHV*) may be a predominant component of the suppression phenotype. Together these mutant analyses provide genetic evidence that *CNA*, *PHB*, *PHV*, and *REV* together with *ATS* are required to sustain normal integument growth.

ATS and *HD-ZIPIII* genes interact to negatively regulate *INO* expression

Of all five *HD-ZIPIII* genes, only *REV* has been shown to be expressed in both integuments (Sieber et al., 2004). Given this difference in expression domains, we wanted to examine roles of *REV* in the absence of *ATS*. We crossed *phb-6 phv-5 rev-9/+* plants, which have wild-type ovules (Figure 4a, b), with *ats-3* (Figure 4c, d) in order to examine whether or not these genes act in the same genetic pathway during integument development. In the segregating F_2 progeny from an *ats-3/+ phb-6/+ phv-5/+ rev-9/+* parent we were able to evaluate the phenotypes of desired mutant combinations after PCR-based genotyping. These mutant studies suggest that *PHB*, *PHV* and *REV* may have overlapping activities with *ATS*, as *ats phv* (Figure 4e, f), *ats phb* (Figure 4g, h) and *ats phb phv* (Figure 4i, j) mutants all show an intermediate phenotype toward symmetrical integument growth (compare Figure 4f, h, j to 4l). Additionally, *ats phb phv rev/+* ovules are more severely affected than *ats phb phv* ovules (compare Figure 4k to 4i, and 4l to 4j), and exhibit completely symmetrical outer integument development (Figure 4h, l). These mutant combinations also have significantly reduced seed set compared to wild type (compare Figure S2d to S2e; S2f). Furthermore, suppression of *ats* does not occur in the *ats phb phv rev/+* mutant, which is in contrast to the *ats phb phv cna* quadruple mutant (Figure 3). This phenotypic difference suggests that both *CNA* and *REV* may act in concert with *PHB* and *PHV* in different manners during ovule development. While this hypothesis is consistent with previously demonstrated differences in function between *CNA* and *REV* (Prigge *et al.*, 2005) it is important to note that it is based on our limited ability to examine only partial loss of REV function in the absence of *PHB* and *PHV* because true *phb phv rev* triple mutants are seedling lethal (Emery *et al.*, 2003).

The *ats phb phv rev/+* ovule morphology is reminiscent of *superman* (*sup*) ovules, wherein symmetrical growth results from ectopic *INO* expression on the gynoapical side of the developing ovule (Meister *et al.*, 2002). We examined expression of *INO* in wild type, *phb phv rev/+* and *ats phb phv rev/+* ovules to see if similar expression could account for the observed phenotype. As in wild type, *INO* was expressed only on the gynobasal side of *phb phv rev/+* ovules (Figure 4n compared to m). In contrast, *ats phb phv rev/+* ovules showed ectopic *INO* expression on the gynoapical side of the ovule (Figure 4o, arrow) in addition to the normal gynobasal location. The expression pattern of *INO* in *ats* ovules was not found to differ from that in wild type ovules (McAbee *et al.*, 2006), so the observed difference in expression patterns represent a synergistic effect between *ATS* and *REV*, *PHB*, and *PHV*.

ATS expression is unaltered in *phb-1d* ovules

Antagonism between *KAN* and *HD-ZIPIII* genes occurs during leaf development to delineate abaxial-adaxial boundaries (Bowman *et al.*, 2002; Kidner and Timmermans, 2007). In gain of function alleles of *HD-ZIPIII* such as *phb-1d* and *phv-1d*, leaf tissue becomes adaxialized via repression of abaxial factors, and *KAN* expression is reduced (Emery *et al.*, 2003; Eshed *et al.*, 2001; Eshed *et al.*, 2004; Kerstetter *et al.*, 2001). We examined *ATS* expression in *phb-1d* ovules using *in situ* hybridization to see if a similar mechanism may be acting in ovules. In wild type ovules, *ATS* expression is first seen in the boundary cells between the inner and outer integument (Figure 5a) and later becomes restricted to the inner integument (McAbee *et al.*, 2006). *ATS* is expressed normally in *phb-1d* and *phv-1d* ovules (Figure 5b, c and data not shown), implying that at least these *HD-ZIPIIIs* are not sufficient to negatively regulate *ATS* when ectopically expressed during integument development. This suggests that the canonical repressive interactions between *KAN* and *HD-ZIPIII* genes in lateral organs may not be reiterated in ovules, at least with respect to *ATS* and *PHB* or *PHV*.

Ectopic expression of *ATS* can arrest growth of the outer integument

Misexpression of *KAN* genes can lead to arrest of organ growth (Emery *et al.*, 2003; Kerstetter *et al.*, 2001). We used the *LHG4≫OP* system (Liu and Meinke, 1998; Moore *et al.*, 1998) to ectopically express *ATS* across the chalaza during ovule development under the control of the *ANT* promoter, which is active in the region from which both integuments will form (Elliott *et al.*, 1996). A line harboring a P_{ANT}*:LHG4* transgene (Gross-Hardt *et al.*, 2002) was crossed into P_{OP}*:ATS* and the resulting *ANT≫ATS* F_1 plants were evaluated. Two phenotypic classes of ovules were observed in these F_1 plants: wild type (Figure 6b) and *ino*-like (Figure 6c, d). The *ino*-like ovules had an inner integument and a reduced/absent outer integument (Figure 6c, d). The lack of complete penetrance of the *ino*-like phenotype may be due to weak expression from either the *ANT:LHG4* and/or *OP:ATS* transgenes. These data suggest that expression of *ATS* in the chalaza can lead to outer integument arrest, possibly through negative regulation of *INO*, which would be consistent with our mutant studies (Figure 4).

Discussion

Adaxial expression of *HD-ZIPIII* genes in the inner integument

While roles for *KAN* (*ATS, KAN1* and *KAN2*) and *YABBY* (*INO*) genes in ovule development have been described, the functions of adaxial determinants in this process are less well understood. In leaves, *HD-ZIPIII* expression domains are adjacent to *KAN* expression domains and this arrangement promotes laminar growth (Eshed *et al.*, 2001; Kidner and Timmermans, 2007). Using *in situ* hybridization we show that the *HD-ZIPIII* genes *PHB, PHV* and *CNA* were expressed in a polar fashion in the inner integument, with their mRNA accumulating in the cell layer adjacent to the nucellus (which later differentiates into the endothelium). Thus *PHB, PHV,* and *CNA* expression was juxtaposed with *ATS* expression in ovules (Figure 1), consistent with the polarity model proposed by McAbee et al. (2006). In contrast to the other three examined *HD-ZIPIII* genes, *REV* is expressed broadly in both integuments (Sieber *et al.*, 2004b) and thus does not conform to the proposed model.

HD-ZIPIII expression expands abaxially in leaves of *kan* mutants, demonstrating that *KAN* genes negatively regulate *HD-ZIPIII* expression and contribute to confinement of *HD-ZIPIII* expression to adaxial domains (Bowman *et al.*, 2002; Eshed *et al.*, 2001; Eshed *et al.*, 2004; Kerstetter *et al.*, 2001). However, in ovules it appears that *PHB, PHV,* and *CNA* expression patterns are not governed by *ATS* activity because they are unaltered in *ats* ovules (Figure 1).

McAbee et al. (2006) hypothesized that an absence of abaxial function in *ats* mutants created a single adaxial/abaxial boundary in the ovule, rather than two separate boundaries, which led to the formation of a single integument. They further hypothesized that loss of ATS activity could lead to an expansion of the "adaxial" factor(s) expression domain. Our observation that *PHB, PHV,* and *CNA* expression patterns are unchanged in *ats* ovules (Figure 1), conflicts with this later hypothesis. Rather, it appears that loss of the ATS abaxial boundary function appears to be sufficient to produce the observed integument fusion.

Since *ATS* is not responsible for patterning *PHB, PHV,* or *CNA* expression in ovules, what factor(s) could contribute to the difference in their expression patterns from that of *REV* in this structure? In leaves, the patterning of *HD-ZIPIII* mRNA accumulation occurs in part through negative regulation by miR165/166 (Kidner and Timmermans, 2007). In ovules, the *PHB* promoter appears to be active in both integuments based on GUS activity in the *phb-6* enhancer trap line (Figure S4). Because this promoter activity does not match the mRNA distribution pattern (Figure 1 and Sieber et al. (2004b)), miR165/166 could contribute to restricting *PHB* expression in ovules. In addition, the miRNA-resistant *phb-1d* mutant is seen to expand in expression domain relative to the wild-type (Sieber *et al.*, 2004b) further implicating miRNA in regulation of this gene. Although all five *HD-ZIPIII* genes share the miR165/166 recognition sequence, miR166g has been shown to have differential affects on *HD-ZIPIII* transcripts (Williams *et al.*, 2005). Overexpression of miR166g in the *jabba-1d* mutant led to down-regulation of *PHB, PHV,* and *CNA* mRNAs while *REV* expression was increased (Williams *et al.*, 2005). Based on these data, one hypothesis that could account for the differences in *PHB, PHV,* and *CNA* mRNA distributions compared to the *REV* expression domain could be differential sensitivity to miR165/166 action.

The LITTLE ZIPPER (ZPR) proteins, a novel family of leucine zipper-containing proteins, have recently been proposed to negatively influence HD-ZIPIII activity and expression (Kim *et al.*, 2008; Wenkel *et al.*, 2007). The roles of *ZPR* genes in ovules have not yet been evaluated, but differential activity of ZPR proteins on the *HD-ZIPIII* genes provides another hypothesis for the different expression domains of *REV* and *PHB/PHV/CNA.*

HD-ZIPIII genes are required for patterning and growth during ovule development

Loss of *PHB, PHV,* and *CNA* led to abnormal ovule development characterized by arrested or amorphous inner and outer integuments. It is curious that both integuments were affected when expression of these genes was only detected in the inner integument (Figure 1 and Sieber et al. (2004b)). There are several possible explanations for this combination of observations. It is possible that *in situ* hybridization (Figure 1 and Sieber et al. (2004b)) is insufficiently sensitive to detect low levels of *PHB, PHV,* and/or *CNA* mRNA that may be present in the outer integument. Another possibility derives from the order and timing of

integument formation. Inner integument initiation precedes initiation of the outer integument, and development of both structures is coordinated (Schneitz, 1999). If inner integument patterning is unbalanced, it could impact the quality of outer integument growth by a domino effect. This type of non-cell autonomous action has been described for other transcription factors active in ovules, such as *WUSCHEL* (Gross-Hardt *et al.*, 2002). An additional influencing factor could be production/perception of hormones, such as auxin. Recent studies on *ARF6* and *ARF8* indicate that auxin perception and responsiveness contribute to integument formation (Wu *et al.*, 2006). Given that auxin cues during embryogenesis appear to be mediated by KAN and HD-ZIPIII activity via PIN1 localization (Izhaki and Bowman, 2007), the same could be true in ovules, and alterations in inner integument development could alter the hormone environment in ways that would affect initiation and growth of the outer integument.

Reduction in outer integument growth was also observed in both *phb-1d* (McConnell and Barton, 1998) and *phv-1d* (Figure 2g–i), and inner integument defects were observed in *phv-1d* (Figure 2g–i). The over (ectopic) production of the products of these two genes could therefore produce effects that were similar to those resulting from a decrease in *HD-ZIPIII* function (e. g. Figure 2b). This seemingly paradoxical observation can be explained if an appropriate balance between adaxial and abaxial promoting activities is necessary for proper integument growth, and that an imbalance in either direction results in disruption of this process.

Loss of *CNA/PHB/PHV* can suppress both aspects of the Ats^{-} phenotype in an *ats* mutant background (Figure 3). This observation that the simultaneous loss of abaxial and adaxial functions restores inner integument growth and integument separation (Figure 3) indicates that there may be additional ad/abaxial factors that are active in ovules, or that other functions can substitute for the juxtaposition when both classes of factors are absent. Indeed, the loss of *REV* activity in this background reduces inner integument growth, suggesting that *REV* may be one of the factors that can compensate for loss of *CNA*, *PHB*, and *PHV*. That loss of adaxial activity can mitigate effects of loss of abaxial activity is consistent with our hypothesis that an appropriate balance between the levels of these two activities is critical for normal laminar extension of the integuments.

We also observed that ectopic expression of *ATS* in the chalaza during ovule development can lead to arrest of outer integument growth (Figure 6). These data are also consistent with the concept that an appropriate balance between levels of *KAN* and *HD-ZIPIII* functions must be maintained in order to promote integument growth, but other mechanisms are also possible. For example, ectopic *KAN* and *ATS* expression can lead to meristem arrest (Emery *et al.*, 2003; Kerstetter *et al.*, 2001) and unpublished data), and growth arrest may be a general activity of KAN proteins.

A balance model for the ad/abaxial determinants underlying integument morphogenesis

Based on these novel expression and genetic data we have refined our model for integument growth (Figure 7) from what was previously published (McAbee *et al.*, 2006). We had previously proposed that ATS acted in juxtaposition to a hypothetical adaxial function to promote inner integument growth. We can now say with confidence that this adaxial

function includes the activities of the *HD-ZIPIII* family members *PHB*, *PHV*, and *CNA*, which are expressed adaxially in the inner integument (Figure 7a). These expression patterns may be established through a combination of promoter activities, miR165/166 expression and/or ZPR action but are shown to be independent of ATS activity. The adaxial activity also includes *REV* function, even though this gene is such a role for *REV* comes from comparison of *ats cna phb phv rev/+* ovules to *ats cna phb phv* ovules (Figure 3). The loss of one copy of *REV* enhances the *ats cna phb phv* phenotype by negatively impacting inner integument growth. *ATS* is shown to be a critical component of the abaxial function, but we hypothesize the existence of an additional abaxial function because inner integument growth and integument separation are restored by certain mutant combinations with *ats*. These results can be explained by an ab/adaxial juxtaposition model if the model is modified to hypothesize that integument growth depends on a proper balance between abaxial and adaxial activities, rather than the absolute levels of the activities (Figure 7b). The observation that ATS and HD-ZIPIII expression in the inner integument was not altered when either factor was absent show that, in contrast to the situation in leaves, the ab and adaxial function are not mutually suppressive.

The earlier model (McAbee *et al.*, 2006) and the model for leaf development (Bowman *et al.*, 2002; Kidner and Timmermans, 2007) would predict a progressive loss of integument growth with progressive loss of ab/adaxial factors. However, our observation that loss of HD-ZIPIII activity can partially compensate for loss of ATS activity is inconsistent with these models. The revised balance model can explain these results and provide an explanation of how similar phenotypes can result from loss of function and ectopic expression of *HD-ZIPIII* genes. Whether such a balance mechanism is also acting in leaves, or if it represents a further difference between leaves and integuments has yet to be determined. Another aspect of the balance model relates to the coordinated growth of the inner and outer integuments. Loss of apparently inner integument specific gene functions leads to a disruption in outer integument growth (Figure 2, Figure 3 and (McAbee *et al.*, 2006)), suggesting that inner integument growth positively contributes to outer integument growth. A final aspect of the model is that the pattern and growth are self-reinforcing. Once an initial state of appropriately balanced ad/ab definition is achieved it will be subsequently maintained. This could explain the range of phenotypes observed among ovules of individual polarity mutants, where small stochastic variations in the initial levels of determinants could result in different final outcomes.

Patterning roles of *REV* and *ATS* during integument morphogenesis

While a more complete understanding of REV function in ovules is lacking due to the inability to examine *phb phv rev* ovules, we have found that in the absence of *ATS phb phv rev/+* ovules become *sup*-like (Figure 4). We attribute this phenotype to ectopic *INO* expression (Figure 4). Because we do not observe this same phenotype in *ats phb phv cna* ovules, and *REV* is expressed differently from its paralogs in ovules, we propose that *REV* may have a unique function in conjunction with *ATS* in patterning *INO* expression during integument initiation. This mechanism may be connected with *SPOROCYTELESS/NOZZLE* action, which was previously shown to act with *ATS* to regulate *INO* (Balasubramanian and Schneitz, 2002). While *KAN1* and *KAN2* (the only other *KANADI* genes that appear to play a

role in ovule development) also participate in control outer integument formation, expression patterns of these genes in ovules have not been observable to date (Kelley and Gasser, unpublished data).

How leaf-like are integuments?

The fossil record indicates that the origin of ovules was contemporaneous with the origin of leaves (Andrews, 1963; Gasser *et al.*, 1998; Herr, 1995). According to the telome theory, the inner integument is homologous to lateral sterile or sterilized structures (born on a reproductive telome truss, with the nucellus being homologous to the apical fertile telome). The outer integument was gained later in plant history, on the stem lineage leading to angiosperms, possibly by transformation from a leaf-like structure known as a cupule (Doyle, 2006; Gasser *et al.*, 1998).

Separate origins for both integuments are also supported at the molecular level as the control of inner and outer integument development occurs through different genes ((McAbee *et al.*, 2006; Skinner *et al.*, 2004) and this study). For instance, *ATS* and *CNA/PHB/PHV* drive inner integument growth while *INO* and *KAN1/2* regulate outer integument growth, in both cases *REV* is an apparent additional participant (Figure 7). Thus the inner integument shares process homology with leaves, in that it is not directly derived from a leaf but it does utilize the same set of gene classes (*KANADI* and *HD-ZIPIII*) to control development. On the other hand, the outer integument is believed to share structural homology with leaves (being most likely derived from a cupule (Doyle, 2006)). The outer integument developmental genetic program would, therefore, be expected to also include a YABBY, in this case the diverged family member *INO*. If REV acts as the corresponding adaxial factor in the outer integument, it may do so independent of a delineated ad/abaxial expression boundary (Sieber *et al.*, 2004b). Current phylogenetic analyses of these three gene families (*KAN*, *HD-ZIPIII* and *YABBY*) are consistent with these hypotheses, with the origin of *KAN* and *HD-ZIPIII* lineages pre-dating seeds and leaves and *YABBY* genes originating with seed plants (Floyd and Bowman, 2007).

Together, our studies, and those of previous researchers (Balasubramanian and Schneitz, 2002; McAbee *et al.*, 2006; Sieber *et al.*, 2004b), demonstrate that while there are similarities between integuments and leaves, there are also marked differences. Although these organs have distinct evolutionary origins, a common set of polarity determinants appears to have been serially utilized in both sets of structures. The differences in the precise roles and interactions of the determinants in each structure may represent differences from the time of their origin or their derived states resulting from subsequent structural diversifications.

Experimental Procedures

Plant material and cultivation

Arabidopsis plants were grown under long-day conditions as previously described (McAbee *et al.*, 2006). Unless otherwise stated, the alleles used in this study were *ats-3* (McAbee *et al.*, 2006); *phb-6*, *phv-5*, *rev-9* (Emery *et al.*, 2003); and *cna-2* (Prigge *et al.*, 2005). Triple

mutant *cna-2 phb-13 phv-11* seed was a kind gift from Steven Clark (Prigge *et al.*, 2005). *phb-6 phv-5 rev-9/+*, *ANT:LHG4*, and *OP:ATS* seeds were a kind gift from John Bowman.

To create *ats-3 phb-6 phv-5* and *ats-3 phb-6 phv-5 rev-9/+* plants *phb-6 phv-5 rev-9/+* pistils were pollinated with *ats-3* pollen on four independent plants. Ten (10) F_1 plants from each cross were genotyped for *ATS/ats-3*, *PHB/phb-6*, *PHV/phv-5*, and *REV/rev-9* alleles using the polymerase chain reaction (PCR) (see Table S1 for a list of primers used in this study). Three of the F_1 *ats-3/+ phb-6/+ phv-5/+ rev-9/+* plants were allowed to self-pollinate to create segregating F_2 populations. Approximately 360 Basta-resistant (*rev-9* carries a Basta resistance marker) F_2 plants were genotyped for *ATS/ats-3*, *PHB/phb-6*, *PHV/phv-5*, and *REV/rev-9* alleles using PCR. Seeds from these plants were evaluated for *ats-3* or wild type morphology. From this population we identified three *ats-3 phb-6/+ phv-5 rev-9/+* individuals. F_3 (and subsequent generations) *ats-3 phb-6 phv-5* and *ats-3 phb-6 phv-5 rev-9/+* plants were identified by PCR-based genotyping.

To create *ats cna phb phv* and *ats cna phb phv rev/+* plants *cna-2 phb-13 phv-11* pistils were pollinated with *ats-3 phb-6 phv-5 rev-9/+* pollen on two plants. Five F_1 plants were genotyped for *REV/rev-9*; three of the five were *rev-9/+*. These three individuals were further genotyped for *ATS/ats-3*, *CNA/cna-2*, *phb-13/phb-6*, and *phv-11/phv-5*; all three were confirmed to be *ats-3/+ cna-2/+ phb-13/phb-6 phv-11/phv-5*. The resulting segregating F_2 population (193 individuals) from a self-pollinated *ats-3/+ cna-2/+ phb-13/phb-6 phv-11/ phv-5 rev-9/+* plant contained 14 sterile plants and 18 plants with an abnormal seedling phenotype (Figure S3): 1–2 radialized cotyledons; hyperaccumulation of anthocyanins in cotyledons; no shoot apical meristem and swollen hypocotyls. These phenotypes are similar to the previously described *phv rev* and the *phb phv rev* phenotypes (Prigge *et al.*, 2005) and occurred at approximately a 1 in 16 frequency. We completed genotyping on 96 of the remaining 193 individuals for *ATS/ats-3*, *CNA/cna-2*, *phb-6*, *phv-11/phv-5*, and *REV/rev-9*. Among these 96 F_2 plants we identified one *ats cna phb-6 phv* individual and four *ats cna phb-6 phv rev/+* individuals; these individuals were used for phenotypic analyses.

To create an ectopic *ATS* expression line we used the *pOpL* two-component system (Liu and Meinke, 1998; Moore *et al.*, 1998). The *ANT:LHG4* line was previously described (Schoof *et al.*, 2000). The *OP:ATS* line was created by cloning the *ATS* cDNA into the 10-OP BJ36 vector (Moore *et al.*, 1998) using BamHI as the 5′ site and HindIII as the 3′ site; the *OP:ATS* cassette was then cloned into pMLBART as a *NotI* fragment and then transformed by the floral dip method (Clough and Bent, 1998) into Ler (Eshed and Bowman, unpublished). We crossed *ANT:LHG4* into *OP:ATS* to generate *ANT≫ATS* plants. Ovules in the resulting F_1 progeny from 3 different individuals were phenotyped.

DNA Extraction and Genotyping

Genomic DNA was extracted from *Arabidopsis* leaf tissue using 2X CTAB buffer (2% cetyl-trimethyl-ammonium bromide (CTAB), 1.4 M NaCl, 100 mM Tris-HCl pH 8.0, 20 mM EDTA) followed by chloroform extraction and DNA precipitation with isopropanol at room temperature. DNA pellets were washed with 70% ethanol, re-suspended in 100 μl of sterile water and stored at −20°C. Genotyping was performed by PCR with 2.0 μl of

genomic DNA in a 25 μl reaction with either GoTaq Master Mix (Promega, http://www.promega.com) or ExTaq DNA Polymerase (Takara, http://www.takara-bio.com).

Oligonucleotides

Primers used in this study for genotyping are listed in Table S1.

Microscopy

Whole mount clearings were prepared by dissecting ovules from carpels using needles and clearing for 2–3 days in a couple drops of Hoyer's solution (7.5% gum arabic (w/v), 6 M chloral hydrate, 5% (v/v) glycerol) under a coverslip according to Liu & Meinke (1998). Ovules were photographed on a Zeiss (http://www.zeiss.com) Axioplan microscope with Normarski optics using a Zeiss Axiophot camera.

For scanning electron micrography (SEM), tissue was fixed and critical point dried as previously described (McAbee *et al.*, 2006) and imaged on a Philips XL 30 SEM (FEI Company, http://www.fei.com).

Light micrographs were taken on a Kodak DC290 (Kodak, http://www.kodak.com) camera mounted on a Zeiss SV8 stereomicroscope.

All images were acquired digitally and edited in Adobe Photoshop CS2 (http://www.adobe.com).

In situ hybridization

Digoxigenin (DIG)-labeled antisense probes for *in situ* hybridization were synthesized using plasmids purified with a Qiagen Miniprep Kit (Qiagen, http://www.qiagen.com) as previously described (McAbee *et al.*, 2006). For antisense *PHB* probe pGEM-PHB (Williams *et al.*, 2005) was linearized with SphI and transcribed with SP6 RNA Polymerase (Promega). For antisense *PHV* probe pGEM-PHV (Williams *et al.*, 2005) was linearized with NotI and transcribed with T7 RNA Polymerase (Promega,). For antisense *CNA* probe pGEM-CNA (Williams *et al.*, 2005) was linearized with SphI and transcribed with SP6 RNA Polymerase (Promega). For antisense *INO* probe pJMV86 (Villanueva *et al.*, 1999) was linearized with XhoI and transcribed with T7 RNA Polymerase (Promega). For antisense *ATS* probe pBS-KAN4 (a gift of John Bowman) was linearized with HindIII and transcribed with T7 RNA Polymerase (Promega). Tissue fixation and *in situ* hybridization were performed as previously described (McAbee *et al.*, 2006), with the following modifications: inflorescences were fixed in FAA for 2 hours at room temperature prior to dehydration in ethanol and embedding in Paraplast-Xtra (Fisher Scientific, http://www.fishersci.com). Slides were hybridized with approximately 10 pg of DIG-labeled probe overnight at 53°C. Immunological detection of the DIG-labeled probes was performed with a DIG Nucleic Acid Detection Kit (Roche, http://www.roche.com) according to manufacturer's instructions. Following detection, slides were rinsed in sterile water and mounted with Crystal Mount (Fisher Scientific) and a coverslip prior to being photographed under DIC using a Zeiss Axiophot camera attached to a Zeiss Axioplan microscope. Digital images were edited using Adobe Photoshop CS2.

Seed set measurements

Measurements of seed number per silique represent the average values from three individual plants (biological replicates). Each biological replicate is comprised of the average value obtained from five siliques (technical replicates). For statistical analysis of genetic effects, seed number per silique was compared by Kruskal-Wallis one way ANOVA on ranks, with pairwise comparisons (Student-Newman-Keuls Method), using Sigma Stat v3.5 (Systat, http://www.systat.com).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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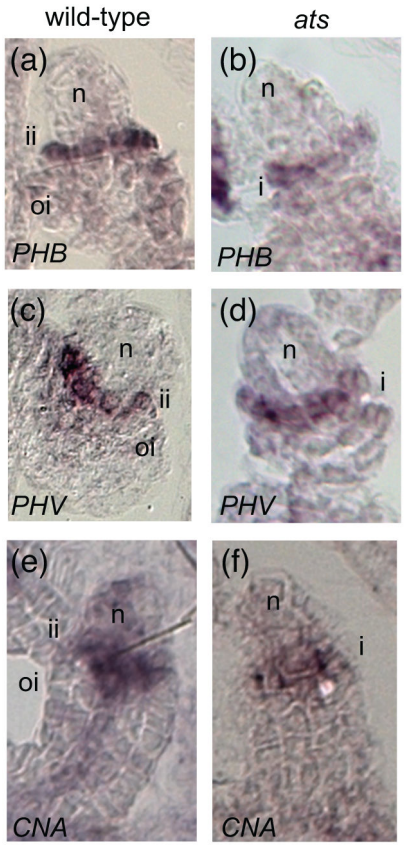

Figure 1.

The polar expression patterns of *HD-ZIPIII* genes are independent of ATS activity. (a), (c), (e) wild-type Ler ovules; (b), (d), (f) *ats-1* mutant ovules; (a), (b) *in situ* hybridization with antisense *PHB* probe. (c), (d) *in situ* hybridization with antisense *PHV* probe. (e) *in situ* hybridization with antisense *CNA* probe. Abbreviations: nucellus (n), inner integument (ii), outer integument (oi).

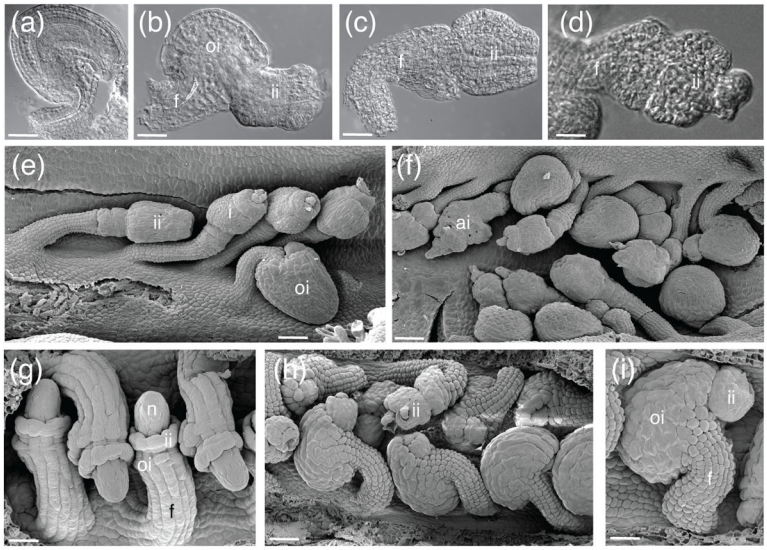

Figure 2.

HD-ZIPIII genes are required for integument morphogenesis. (a)–(f) *cna phb phv* triple mutant ovules; (g)–(i) *phv-1d* gain-of-function ovules. (a)–(d) whole-mount ovule clearings. (e)–(i) SEMs. Scale bar = 20 μm in (a)–(d), (i). Scale bar = 50 μm in (e), (f), (h). Scale bar = 5 μm in G. Abbreviations: nucellus (n), outer integument (oi), inner integument (ii), funiculus (f), amorphous integument (ai), integument (i).

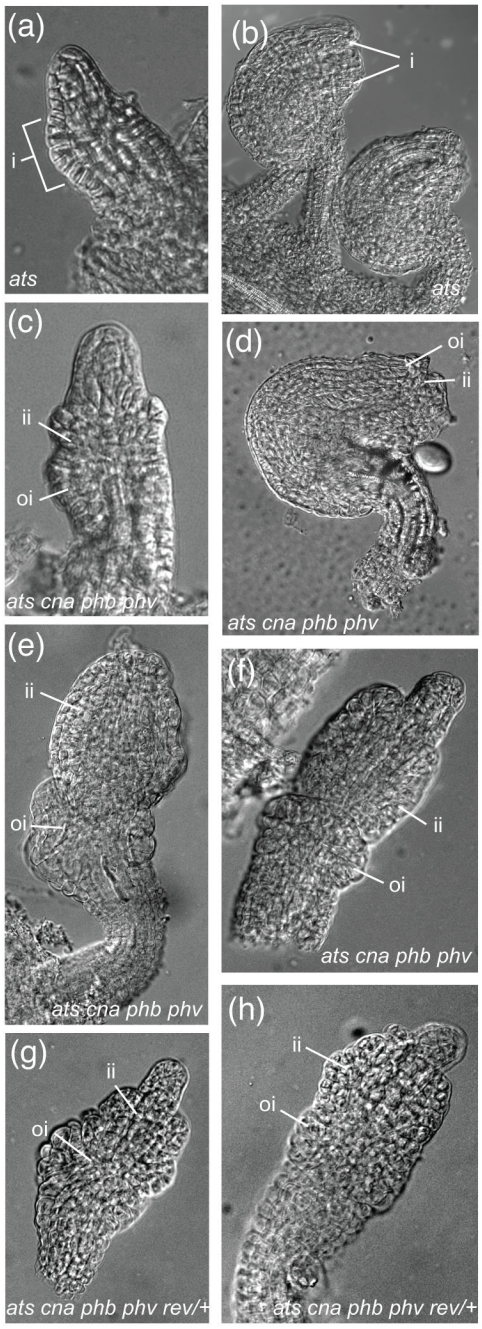

Figure 3.

Loss of function of *CNA*, *PHB*, and *PHV* can suppress the *Ats*$^{-}$ phenotype. (a)–(b) whole-mount clearings of *ats;* (c)–(f) whole-mount clearings of *ats cna phb phv* ovules; (g)–(h) whole-mount clearings of *ats cna phb phv rev/+* ovules. Scale bar = 5 μm in (a), (c). Scale bar = 10 μm in (b), (d)–(h). Abbreviations: nucellus (n), integument (i), inner integument (ii), outer integument (oi), funiculus (f).

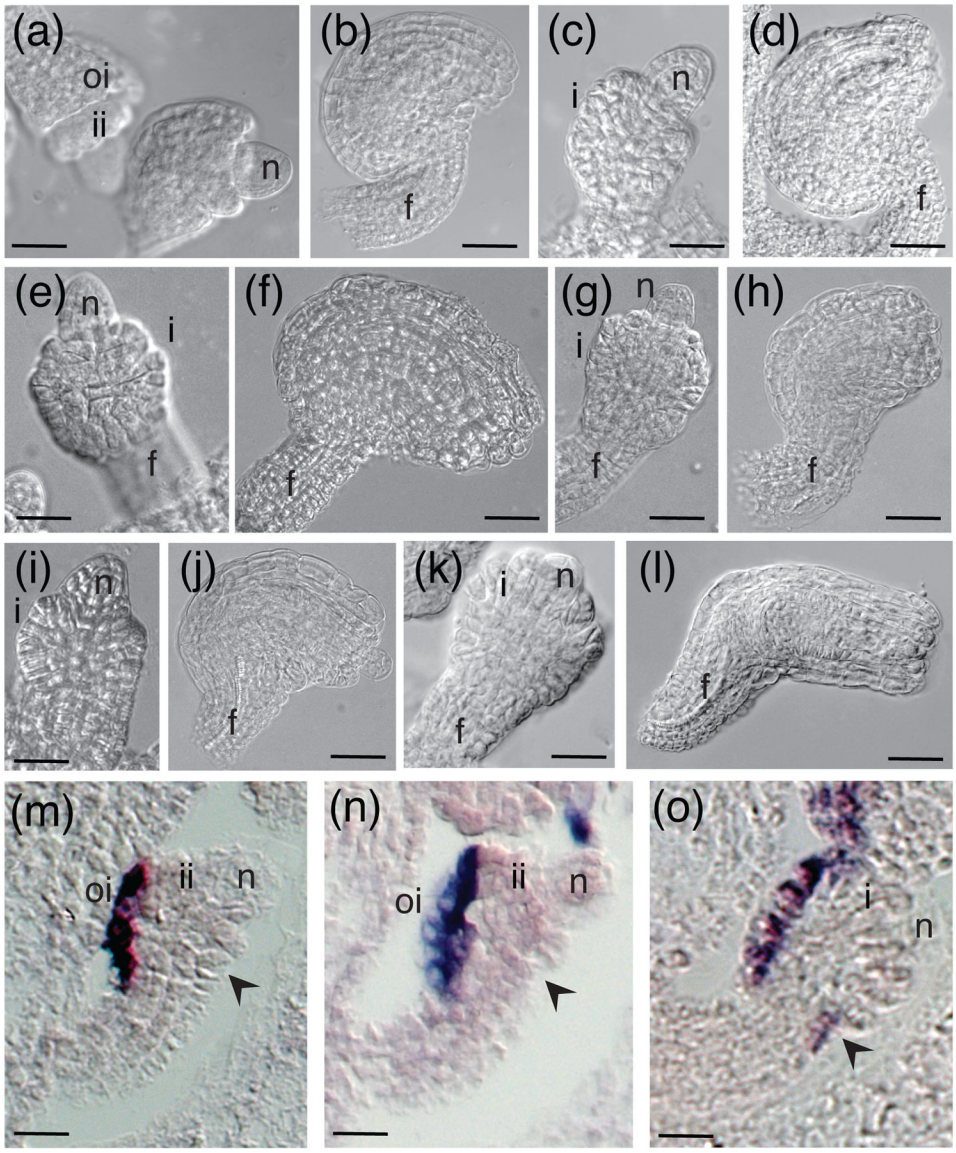

Figure 4.

Genetic interactions between *HD-ZIPIII* genes and *ATS*. (a), (b) *phb phv rev*/+; (c), (d) *ats* single mutant; (e), (f) *ats phv* double mutant; (g), (h) *ats phb* double mutant; (i), (j) *ats phb phv* triple mutant; (k), (l) *ats phb phv rev*/+. (m) wild-type Ler. (n) *phb phv rev*/+. (o) *ats phb phv rev*/+. (a)–(l) whole mount clearings of ovules. (m)–(o) *in situ* hybridizations with antisense *INO* probe. Scale bar = 5 μm in (a), (c), (e), (g), (i), (k), (m)–(o). Scale bar = 10 μm in (b), (d), (f), (h), (j), (l). Black arrow in (m)–(o) indicates the gynoapical side of the ovule. Abbreviations: nucellus (n), inner integument (ii), outer integument (oi), funiculus (f), integument (i).

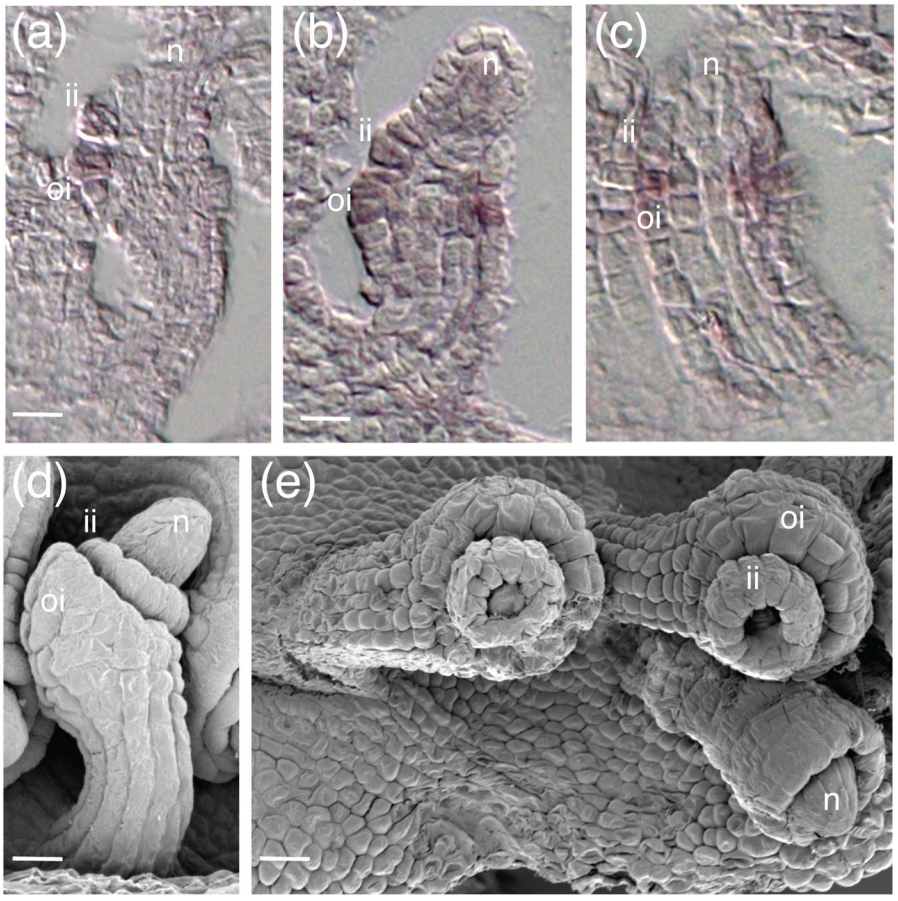

Figure 5.

ATS is expressed normally in *phb-1d* mutant ovules. (a), (d) wild-type Ler ovules; (b), (c), (e) *phb1d* ovules. (a)–(c) *in situ* hybridizations with antisense *ATS* probe. (d)–(e) SEMs. Scale bars = 5 μm. Abbreviations: nucellus (n), inner integument (ii), outer integument (oi).

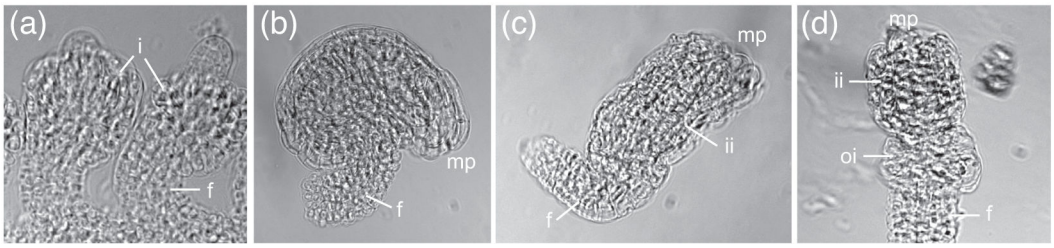

Figure 6.

Ectopic expression of *ATS* can arrest growth of the outer integument. (a)–(d) whole-mount clearings of *ANT≫ATS* ovules. (a) integument initiation. (b)–(d) mature ovule phenotypes. (b) wild-type ovule. (c)–(d) *ino*-like ovules. Abbreviations: integuments (i), inner integument (ii), outer integument (oi), micropyle (mp), funiculus (f).

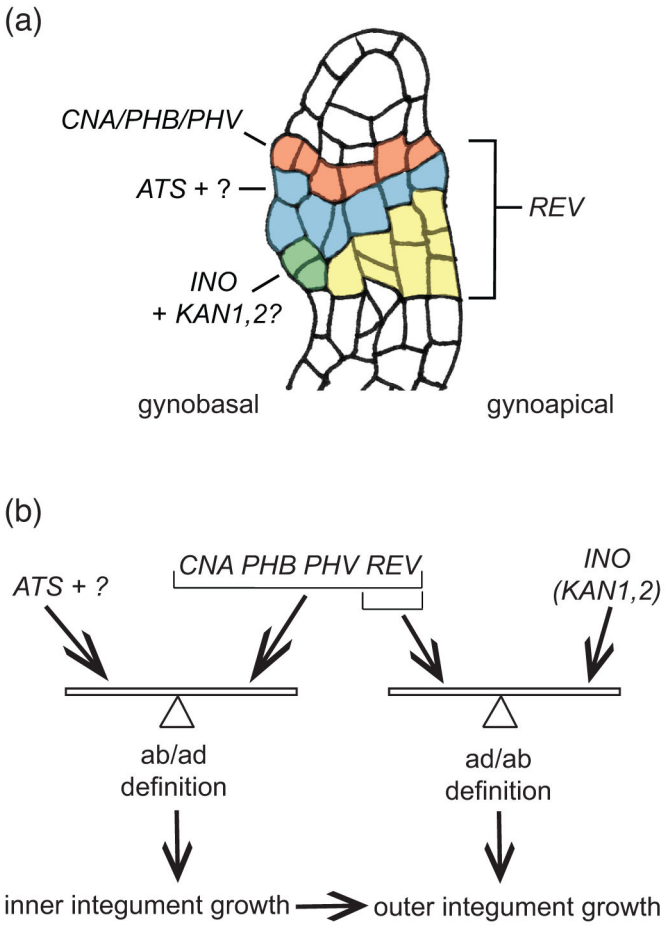

Figure 7.

The collective roles of polarity determinants in ovule development comprise the balance model. (a) Expression summary of polarity determinants required for integument growth. During early ovule development *CNA*, *PHB*, and *PHV* (red), *REV* (all colored regions), *ATS* (blue), "?" unknown abaxial factor(s) which may share expression domain with *ATS*, and *INO* (green) mRNAs are specifically patterned along the chalaza. *KAN1* and *KAN2* (green) are predicted to be expressed in a similar fashion to *INO*. Once these patterns are established, persistent expression/action of all these transcription factors is required for proper integument growth. (b) The proposed polarity balance model. In the inner integument, *ATS* and the proposed additional abaxial factor ("?") act in balanced opposition to *CNA/PHB/PHV* and *REV* to promote inner integument growth. Disruption of this balance by decrease or increase in either function alters the growth plane, leading to aberrant integument growth. Initiation of the inner integument precedes outer integument initiation in *Arabidopsis*, and thus growth of the outer integument is directly influenced by inner integument morphogenesis. In the outer integument, abaxial activities provided by *INO*, *KAN1*, and *KAN2* act in balance with *REV* to control morphogenesis.