4291

NOZZLE links proximal-distal and adaxial-abaxial pattern formation during ovule development in *Arabidopsis thaliana*

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SUMMARY

The ovules of *Arabidopsis* show polarity along the proximal-distal and the adaxial-abaxial axis. *NOZZLE,* a gene that encodes a novel protein and *BELL1,* encoding a homeodomain protein, play a vital role in pattern formation along the proximal-distal axis. *INNER NO OUTER,* which encodes a member of the YABBY family of transcription factors and *SUPERMAN*, encoding a zinc finger transcription factor, are essential for the establishment and maintenance of adaxial-abaxial polarity. To date, the co-ordination of patterning along these two axes is unclear. Here we show that *NOZZLE* plays a vital role in pattern formation along the adaxial-abaxial axis as well. We investigated the expression of *INNER NO OUTER* in various mutant backgrounds and have identified *ABERRANT TESTA SHAPE* and *NOZZLE* as spatial regulators of *INNER NO OUTER* expression. In addition, we show that *NOZZLE* and *AINTEGUMENTA,* which encodes an AP2 domain transcription factor, regulate the temporal expression of *INNER NO OUTER* and that

BELL1 is essential for *INNER NO OUTER* expression. We further analysed the expression of *BELL1* and *AINTEGUMENTA* in *inner no outer* mutants and show that the positive auto-regulatory control of *INNER NO OUTER* expression involves *AINTEGUMENTA*. Based on our results we propose a model for adaxial-abaxial pattern formation during ovule development. Our results indicate that *NOZZLE* plays a central role in patterning both the proximal-distal and the adaxial-abaxial axes. Furthermore, negatively regulating *INO* expression in a temporal manner, ensures that the adaxial-abaxial polarity is established after the specification of the chalaza, a proximal-distal axis pattern element. It therefore serves as a molecular link between these processes during ovule development in *Arabidopsis thaliana*.

Key words: Ovule development, Pattern formation, Organogenesis, *Arabidopsis thaliana, NOZZLE, INNER NO OUTER*

INTRODUCTION

Organogenesis, the formation of an organ from a group of undifferentiated cells, is a precisely controlled process that results in a structure of a specific size and shape. This process involves several sub-processes such as establishment of identity, initiation and outgrowth, pattern formation and morphogenesis. Achievement of the proper size and shape of an organ requires orchestration of all these activities and this represents one of the basic questions in developmental biology. Ovules, the progenitors of seeds, are the female reproductive organs of higher plants. In *Arabidopsis thaliana,* they provide an excellent model system to study organogenesis (Gasser et al., 1998; Grossniklaus and Schneitz., 1998; Chevalier et al., 2002). In *Arabidopsis*, ovules develop from the placenta within the gynoecium composed of two fused carpels. They arise as finger-like protrusions, which are radially symmetrical. Yet, at maturity, ovules of *Arabidopsis* show polarity in at least two axes of symmetry. Along the proximal-distal (PD) axis, three morphologically distinct units can be observed in an ovule

(Esau, 1977; Schneitz et al., 1995). The nucellus at the distal end harbours the megaspore mother cell (mmc) that undergoes meiosis to eventually form the embryo sac. From the central region, referred to as chalaza, two integuments initiate that eventually envelop the nucellus and the growing embryo sac. Proximally, the funiculus connects the ovule to the placenta. *Arabidopsis* ovules also show polarity along the adaxial-abaxial (Ad-Ab) axis. The initiation of the outer integument from the abaxial epidermis of the proximal chalaza visibly marks the first sign of the polarity along the Ad-Ab axis in a developing ovule. The outer integument shows differences along the Ad-Ab axis not only in its initiation but also in its growth, as it grows more on the abaxial side than on the adaxial side. This high level growth on the abaxial side forces a curvature in the developing ovule, which in part results in its anatropy (Robinson-Beers et al., 1992; Schneitz et al., 1995).

How are these patterns set up and what are the underlying molecular mechanisms? Genetic and molecular analysis have identified several genes that play a role in pattern formation along these two axes. *BELL1 (BEL1)* encodes a homeodomain

protein and *bel1* mutants show abnormal outgrowths in place of integuments (Modrusan et al., 1994a; Ray et al., 1994; Reiser et al., 1995; Schneitz et al., 1997). *BEL1* is expressed throughout the ovule primordia in the initial stages, but is restricted to the central region before the initiation of integuments, and thus marks the central region at a molecular level (Reiser et al., 1995). The biochemical nature of the BEL1 protein, the expression pattern of *BEL1* and the *bel1* phenotype make *BEL1* an excellent candidate for patterning the PD axis. Recently we have reported the genetic analysis of *NOZZLE (NZZ)* and shown that *NZZ* functions redundantly with *BEL1* in specifying the chalaza (Balasubramanian and Schneitz, 2000). In the ovules of *nzz bel1* double mutants no chalaza structures are detectable and the tissue that is formed in the central region resembles funiculus as seen by the epidermal cell morphology. *NZZ* encodes a novel protein that plays a role in both male and female reproductive development (Balasubramanian and Schneitz, 2000; Schiefthaler et al., 1999; Yang et al., 1999). *NZZ* shows an antagonistic genetic interaction with *AINTEGUMENTA (ANT),* which encodes an AP2 domain-containing transcription factor (Elliott et al., 1996; Klucher et al., 1996). *ANT* controls cell proliferation and organ size during ovule and flower development (Krizek, 1999; Krizek et al., 2000; Liu et al., 2000; Mizukami and Fischer, 2000; Schneitz et al., 1998). From our previous genetic analysis of *nzz,* we proposed that *NZZ,* through its interactions with *BEL1* and *ANT,* couples PD pattern formation and growth during ovule development in *Arabidopsis thaliana* (Balasubramanian and Schneitz, 2000).

What regulates the Ad-Ab pattern formation? Studies in several species have identified many loci that regulate Ad-Ab patterning during lateral organ formation. Analysis of mutants like *phantastica (phan)* of *Antirrhinum, leafbladeless (lbl1)* of maize, *lam1* of *Nicotiana* and *argonaute (ago1), pinhead/zwille (pnh/zll), phabulosa (phb)* and *phavoluta (phv)* of *Arabidopsis* have shown that the corresponding wild-type genes promote adaxial cell fate (Bohmert et al., 1998; Lynn et al., 1999; McConnell and Barton., 1998; McConnell et al., 2001; McHale and Marcotrigiano, 1998; Timmermans et al., 1998; Waites and Hudson, 1995). Contrary to this, the members of the *YABBY* gene family, which encode transcription factors, and the *KANADI* genes, which also encode transcription factors, promote abaxial cell fate (Bowman, 2000a; Eshed et al., 1999; Golz and Hudson, 1999; Kerstetter et al., 2001; Siegfried et al., 1999). The members of the *YABBY* gene family are expressed in a polar manner at the abaxial side of lateral organs. *KANADI* genes redundantly promote abaxial cell fate possibly by negative regulation of the *PHB/PHV* mediated adaxial signaling. When *KANADI* function is compromised, adaxialised organs are formed (Eshed et al., 2001).

INNER NO OUTER (INO), a member of the *YABBY* family plays a vital role in Ad-Ab pattern formation during ovule development (Villanueva et al., 1999). *ino* mutants exhibit ovules that lack the outer integument (Baker et al., 1997; Schneitz et al., 1997) and *INO* expression is detected in cells that give rise to the outer integument before its initiation (Villanueva et al., 1999). Therefore, it has been implicated in the establishment and maintenance of this axis in ovules. *SUPERMAN (SUP),* a gene that encodes a zinc finger transcription factor, is another locus that regulates the Ad-Ab

pattern formation (Gaiser et al., 1995; Sakai et al., 1995). Interestingly in *sup* mutants, the outer integument initiates properly, but grows equally on both adaxial and abaxial side suggesting that *SUP* may be necessary for the maintenance rather than the initial establishment of the Ad-Ab axis.

The orchestration of cell activities along the various axes of polarity is crucial for the proper development of any organ. How is the co-ordination of cell activities along the PD and Ad-Ab axis achieved during ovule development? What is the molecular link between patterning along these two axes? How is the expression of *INO* regulated in a spatial and temporal manner to ensure that such a co-ordination is achieved? Here we report the expression patterns of *INO* in various mutant backgrounds and show how its transcription is regulated in a spatial and temporal manner. We show that the co-ordination of *BEL1, ANT* and *NZZ* activities is required for the onset and temporal expression of *INO*. We show that at least three genes *NZZ, ATS* and *SUP* regulate the spatial expression of *INO*. We present evidence that *NZZ* and *ATS* spatially restrict the expression of *INO* to the abaxial epidermis. We further report the expression patterns of *ANT* in *ino* and *nzz ino* double mutants and show that the positive auto-regulatory control of *INO* expression (Villanueva et al., 1999) involves *ANT*. We propose a model that summarises our findings and explains how Ad-Ab patterning and outer integument development occurs during ovule development in *Arabidopsis*. Our analysis indicates *NZZ* as a molecular link that orchestrates pattern formation along PD and Ad-Ab axis during ovule development in *Arabidopsis*.

MATERIALS AND METHODS

Plant growth and mutant alleles

Plants were grown as described previously (Balasubramanian and Schneitz, 2000; Schneitz et al., 1997) and *Arabidopsis thaliana* (L) Heynh. var. Landsberg (*erecta* mutant) was used as a wild-type strain. For the double mutant analysis and in situ expression analysis the following mutant alleles were used: *nzz-2, ant-72F5, bel1-1460, ats, ino-2* and *sup-5*. All these mutant alleles have been described previously. *nzz-2* is a putative null (Schiefthaler et al., 1999). *ant-72F5, bel1-1460* show a strong phenotypes comparable to null alleles of *bel1* and *ant* (Schneitz et al., 1997). The molecular nature of the single *ats* allele is unknown since *ATS* has not yet been cloned (Lèon-Kloosterziel et al., 1994). *ino-2* is a strong allele and has a defect that leads to alternate splicing which in turn results in addition of 11 nucleotides leading to a frame shift. This addition is unlikely to cause a defect in mRNA stability (Villanueva et al., 1999). *sup-5* shows the strong ovule phenotype and has been described previously (Gaiser et al., 1995). *nzz-2 ats* double mutants were recognised by their novel phenotype in the expected segregation ratio. More than 20 double mutant plants were analysed.

Scanning electron microscopy (SEM) and in situ hybridisation

SEM and image processing has been described previously (Balasubramanian and Schneitz, 2000; Schiefthaler et al., 1999; Schneitz et al., 1998; Schneitz et al., 1997). The protocol for in situ hybridisation and the *INO, BEL1* and *ANT* probes that were used in these experiments have also been described previously (Balasubramanian and Schneitz, 2000). In situ experiments were repeated several times, with different batches of fixed material to rule out the possibility of a negative result due to experimental or material batch differences. Furthermore, the sections of wild-type and the

mutant tissues were processed together in order to minimise experimental differences.

RESULTS

Wild-type ovule development

Ovule development in wild-type *Arabidopsis* is very well documented (Modrusan et al., 1994b; Robinson-Beers et al., 1992; Schneitz et al., 1995). Here we present a brief overview of the morphological distinctions that are visible along the PD and Ad-Ab axis during development. Ovules arise as finger-like protrusions from the placental tissue of the carpels (Fig. 1A). Around stage 2-I, a hypodermal cell enlarges and differentiates into the mmc at the distal end thus marking the nucellus. The ovule primordia appear radially symmetrical at this stage (Fig. 1A). The integuments show a temporal difference in their initiation. The inner integument initiates earlier than the outer integument. Around stage 2-II, the initiation of the inner integument takes place in a symmetrical manner from the distal chalaza (Fig. 1B). The establishment of the Ad-Ab axis is visible with the initiation of the outer integument at about stage 2-III (Fig. 1B). Few cells in the abaxial epidermis of the proximal chalaza show a bulge at this stage. An enlarged epidermal cell undergoes a cell division that produces a triangular tip cell. Subsequent division of the two cells adjacent to this tip cell lead to two cell layers of the outer integument: the outer (abaxial) cell layer and the inner (adaxial) cell layer. The cells of the abaxial layer are more vacuolated than those of the adaxial cell layer (Schneitz et al., 1995). In addition, the abaxial cell layer grows more to encompass the adaxial cell layer. Around stage 3-I, the outer integument envelops the nucellus and the inner integument (Fig. 1C) and further development results in the anatropy that can be observed at maturity (Fig. 1D). The ovule is connected to the placenta through the funiculus that carries a vascular strand.

Outer integument development in *nzz-2, ats, nzz-2 ats* and *sup-5*

Ovule development in *nzz-2, ats* and *sup-5* has been described previously (Balasubramanian and Schneitz, 2000; Gaiser et al., 1995; Léon-Kloosterziel et al., 1994; Schiefthaler et al., 1999). Here we present a brief summary of events that are relevant to our discussions below. *nzz* mutants show pleiotropic defects during ovule development (Balasubramanian

and Schneitz, 2000). The outer integument initiates earlier than the inner integument and sometimes both integuments are reduced (Fig. 1F,G arrow). In *ats* mutants both integuments initiate but the spacing between the integuments is reduced (Fig. 1J). They soon fuse and develop as a single integument, as a result of which, at maturity the nucellar and chalazal regions appear 'round' compared to wild type (Fig. 1K,L). In contrast to the single mutants of either *nzz-2* or *ats, nzz-2 ats* double mutants show drastic differences in the development of the outer integument. Similar to *nzz-2* mutants, the outer integument is initiated earlier than the inner integument (Fig. 1N) in *nzz-2 ats* double mutants. After initiation, the outer integument starts to grow on both the adaxial and the abaxial
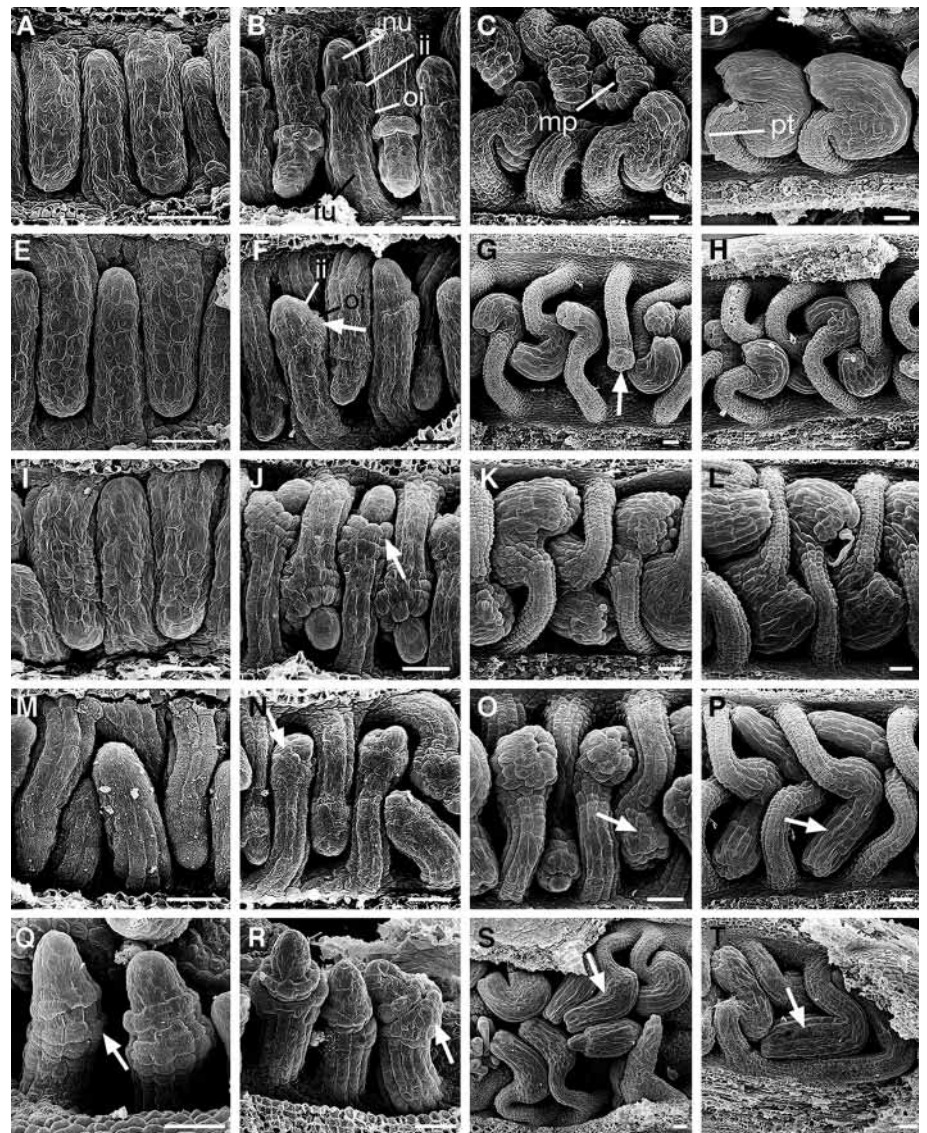
Fig. 1. Ovule development in wild type (A-D), *nzz-2* (E-H), *ats* (I-L), *nzz-2 ats* (M-P) and *sup-5* (Q-T). Stages: (A,E,I,M) 2-I; (B,F,J,N,Q) 2-III; (R) 2-IV; (C,G,K,O,S) 3-IV; (D,H,L,P,T) 4-IV. The outer integument initiates earlier than the inner integument and is visible before the inner integument in *nzz-2* (arrow in F). Adaxial growth of the outer integument can be seen in *nzz ats* (arrows in O and P). Abaxial initiation of the outer integument (arrows in Q and R) and the abnormal growth (arrows S and T) on the adaxial side can be seen in *sup-5*. nu, nucellus; ii, inner integument; oi, outer integument; fu, funiculus; mp, micropile; pt, pollen tube; ad, adaxial; ab, abaxial. Scale bars: 20 μm.

Fig. 2. Expression pattern of *INO*
during ovule development in wild type
(A-D), *ant-72F5* (E-H), *ino-2* (I-L) and
bel1-1460 (M-O). P is a control section
hybridised with the sense probe of *INO*.
Stages: (A,E,I,M) 1-I/II; (B,J,N,P) 2-I;
(F) 2-II; (C,D,K) 2-III; (O) 2-IV; (G,L)
3-I; (H) 4-V. *ant* mutants were staged
on the basis of their ovule, carpel and
anther developmental profile relative to
each other. *INO* expression can be
detected in cells that give rise to the
outer integument in wild type. The cell
that undergoes a division to form two
cell layers and the triangular tip cell
does not express *INO* (arrow in C).
Note the absence of *INO* expression in
the adaxial cell layer (arrow in D). Note
the absence of *INO* expression even at
the site of outer integument initiation in
ant-72F5 (arrow in G). *INO* expression
can be detected at late stages (H,b) but
only in a few epidermal cells, similar to
the early stages in wild type. The
typical horseshoe appearance is not
present (arrow H,a). No *INO* expression
can be detected in *ino-2* (I-L) and *bel1-
1460* (M-O). Arrows in L indicate site
of outer integument initiation in wild
type. Arrows in O denote early chalazal
bulges in *bel1-1460*. Sense probe gave
no signals above background (P). Scale
bars: 20 µm.
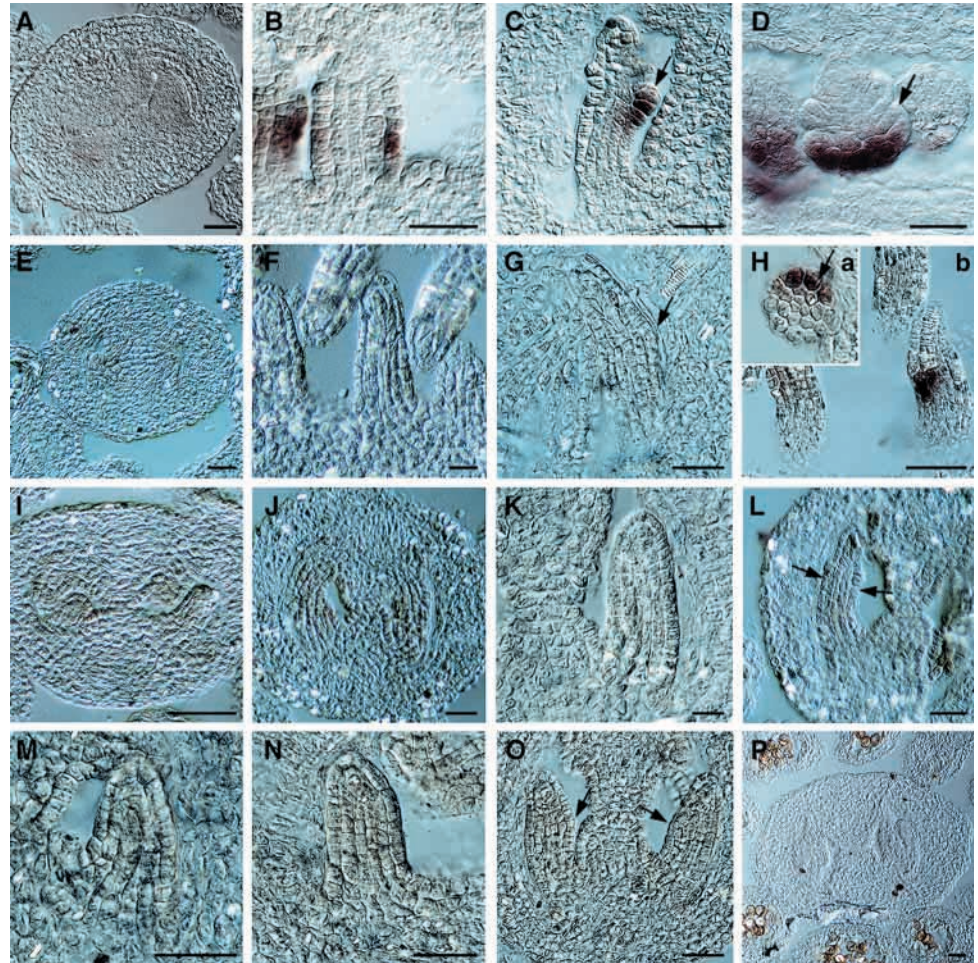
side and fails to show the growth differences usually seen in
wild type (Fig. 1O). Around stage 3-I, the growth of the outer
integument in the adaxial side is clearly visible (Fig. 1O).
Further development of the outer integument in both adaxial
and abaxial side eventually results in a non-anatropous ovule
that looks similar to the ovules of *sup-5*mutants (compare Fig.
1P and 1T). The outer integument development in *sup-5* is very
similar to that observed in *nzz-2 ats* double mutants (Fig. 1Q-
T).

Expression patterns of *INO* in wild type, *ant-72F5*, *ino-2* and *bel1-1460*

Mutations in the *INO* locus lead to lack of the outer integument
(Baker et al., 1997; Schneitz et al., 1997). *INO* expression can
be detected in cells that will give rise to the outer integument,
before its initiation, and therefore it is the earliest molecular
manifestation of the Ad-Ab polarity (Villanueva et al., 1999).
ino exhibits complex genetic interactions and several putative
regulators of *INO* expression have been reported. *BEL1*, *ANT*,
HUELLENLOS (HLL) and *SUP* were suggested to be negative
regulators of *INO* expression (Villanueva et al., 1999). In
contrast, we have previously reported the absence of *INO*
expression in *bel1-1460* and *ant-72F5* mutants at about stage
2-II (Balasubramanian and Schneitz, 2000). In order to
understand the regulation of *INO* expression and outer
integument development, we undertook to analyse *INO*

expression in wild type and various mutants during various
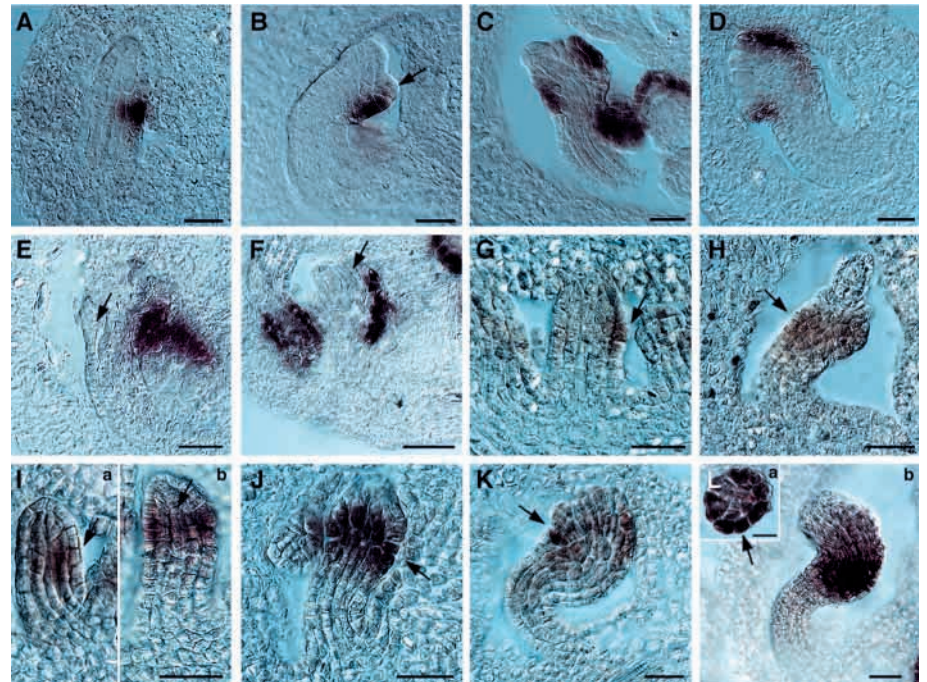stages of ovule development.

Expression of *INO* during wild-type ovule development

INO expression could be first detected at about stage 2-I (Fig.
2B). Around stage 2-III, when the outer integument initiation
becomes visible, *INO* expression is observed in the epidermal
cells that enlarge (Fig. 2C). Interestingly, the cell that
undergoes cell division to give rise to the triangular tip cell and
subsequently the two cell layers, does not express *INO* (Fig.
2C arrow). Later during development, *INO* expression is
observed only in about 3-4 cells at the distal end of the abaxial
cell layer of the outer integument. The adaxial cell layer of the
outer integument does not show any *INO* expression (Fig. 2D).

Expression of *INO* during ovule development in *ant-72F5, ino-2* and *bel1-1460*

Since *ant* and *bel1* are genetically epistatic to *ino* (Baker et al.,
1997), we tested whether *ANT* and *BEL1* are required for the
temporal and spatial expression of *INO* by analysing *INO*
expression in ovules of *ant-72F5* and *bel1-1460*. In *ant-72F5*
ovules, *INO* expression could not be detected until stage 3-1
(Fig. 2E-G), and is detected only at about stage 4-V (Fig. 2H).
The expression is restricted to a few epidermal cells similar to
the wild-type expression of *INO* around stage 2-III. In ovules

Fig. 3. Expression pattern of *INO* during ovule development in *nzz-2* (A-D), *ats* (E,F), *sup-5* (G,H) and *nzz-2 ats* (I-L). Stages: (A,G,I) 1-II/2-I; (B,E,H,J) 2-III; (C) 2-IV; (D,F,K) 3-I; (L) 3-IV. *INO* expression remains unaltered in *nzz-2* except that it might be shifted distally by few cells as observed by the shifting of the outer integument (A-D). The tip cell does not show *INO* expression as in wild type (B arrow). The absence of *INO* expression at the adaxial side can be seen in *ats* mutants (arrow in E). The tip cell and the adaxial cell layer of the outer integument do not show *INO* expression in *ats* mutants (arrow in F). In *sup-5* early onset of *INO* expression is similar to wild type (arrow in G), but around stage 2-III, *INO* expression can be detected throughout the central region (arrow in H). In ovules of *nzz-2 ats* mutants, *INO* expression is similar to wild type in most instances (arrow in I,a), but sometimes the expression can be seen throughout the central region (arrow in I,b). Instead of a 'horseshoe appearance' a complete ring can be observed in a cross section through the central region of a *nzz-2 ats* double mutant (arrow in L,a). Scale bars: 20 µm.

of *bel1-1460, INO* expression could not be detected at any stage during development (Fig. 2M-O). We tested the expression of *INO* in *ino-2* mutants as well. We could not detect any expression of *INO* in *ino-2* at any stage in development (Fig. 2I-L) in accordance with the findings of Villanueva et al. (Villanueva et al., 1999).

Expression of *INO* during ovule development in *nzz-2, ats, sup* and *nzz-2 ats*

We tested if the differences that we observed in the outer integument development in *nzz-2 ats* double mutants were reflected in a change in *INO* expression by analysing its expression in *nzz-2, ats* and *nzz-2 ats* mutant backgrounds. In *nzz-2,* the expression of *INO* was detected at

the site of outer integument, before its initiation (Fig. 3A). The outer integument initiates earlier in *nzz* mutants (Fig. 1F), suggesting precocious *INO* expression in *nzz*. With respect to
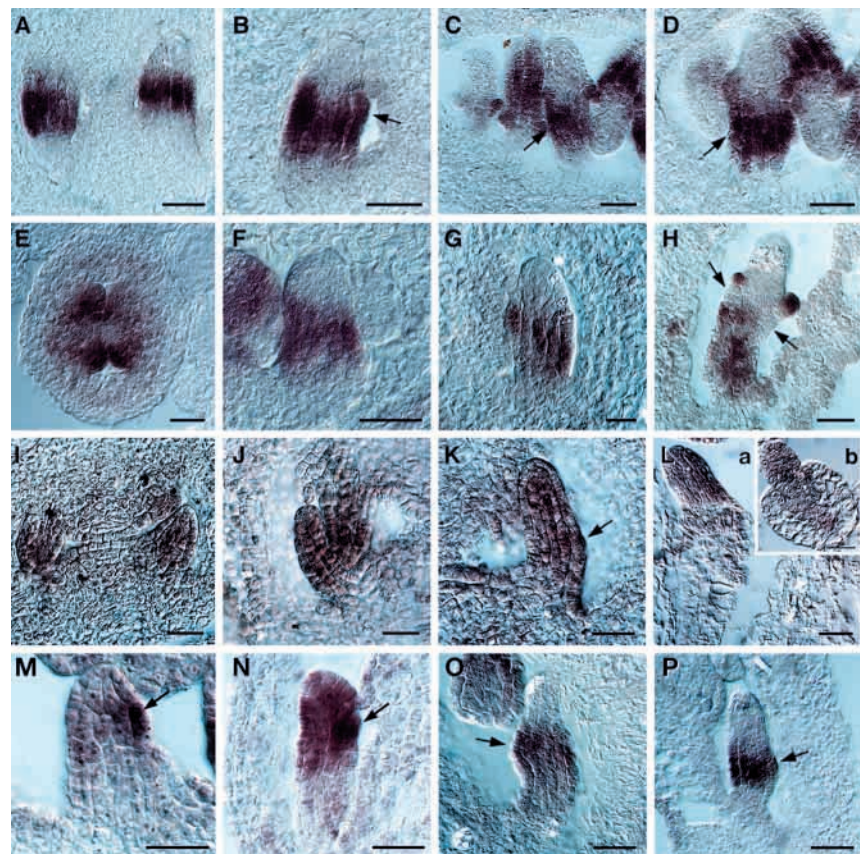
Fig. 4. Expression of *BEL1* and *ANT* in *ino-2, nzz-2 ino-2. (*A-D) *BEL1* expression in *ino-2*. (E-H) *ANT* expression in *ino-2*. (I-L) *ANT* expression in *nzz-2 ino-2*. (M,N) *ANT* expression in *nzz-2*. (O) *ANT* expression in *bel1-1460*. (P) *BEL1* expression in *ant-72F5*. Stages: (E,I) 1-I/II; (A,F,J,M) 2-I; (B,G,N) 2-II; (C,K) 2-III; (D,H,O,P) 2-IV; (L) 3-IV. Note the presence of *BEL1* expression at the site of outer integument initiation in *ino-2* (arrows in B, C and D). Around stage 2-IV, an absence of *ANT* expression can be observed at the site of outer integument initiation in *ino-2* (arrows in H). No strong spot of *ANT* expression can be detected in *nzz-2 ino-2* (arrow in K). Note the presence of *BEL1* expression in *ant-72F5* (P, arrow) and *ANT* expression in *bel1-1460* (O, arrow). Scale bars: 20 µm.

Fig. 5. A genetic model for adaxial-abaxial pattern formation and outer integument development during ovule development in *Arabidopsis thaliana.* (A). Initiation of *INO* expression. *ANT* and *NZZ* act antagonistically and are needed for the correct timing of the onset of *INO* expression. *BEL1* is a pre-requisite for *INO* expression. (B) Feedback regulation of *INO* expression. After initiation, *INO* positively regulates *ANT* expression, which in turn leads to positive regulation of *INO* and *NZZ* expression. *NZZ* in turn forms a negative feedback on *ANT* thereby maintaining the levels of *ANT* and *INO* expression. (C) Spatial regulation of *INO* expression. The dashed lines with a question mark indicate that the interaction between *NZZ, ATS* and *SUP* is unclear. Lines with arrows indicate activation of transcription. Lines with barred ends represent an inhibitory input.
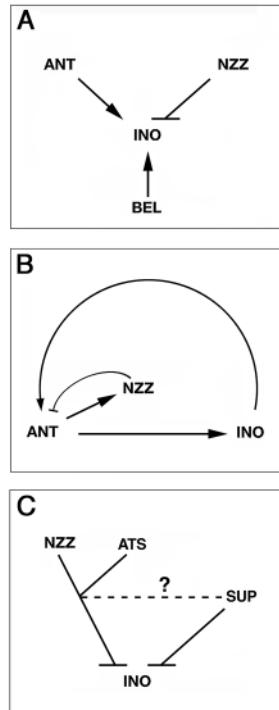
the Ad-Ab axis, the expression of *INO* in *nzz-2* showed no deviation from wild type (Fig. 3B-D). In addition, *INO* expression could easily be detected with shorter colour reaction times (about 12 hours) in *nzz-2* compared to wild type (about 36 hours) hinting at an increased level of *INO* expression in *nzz-2*. In *ats* mutants, *INO* expression followed a wild-type expression pattern (Fig. 3E,F). In contrast to the single mutants of both *nzz-2* and *ats, nzz-2 ats* double mutants showed drastic alterations in the expression pattern of *INO* during ovule development. At initial stages, the normal *INO* expression pattern was observed, though a weak signal was detected throughout the central region (Fig. 3Ia). Sometimes, it was more obvious, with *INO* expression detected throughout the central region similar to a *BEL1* or *ANT* stripe (Fig. 3Ib). This was even more pronounced at around stage 2-III and all the ovules that we observed (*n*>40) showed the expression of *INO* throughout the central region (Fig. 3J). Later, *INO* expression was detected on both the adaxial and the abaxial side of the ovule and in both the adaxial and abaxial cell layers of the outer integument (Fig. 3K). Even around stage 3-IV, *INO* expression was detected throughout the central region and was not restricted to the epidermis (Fig. 3La). The misexpression of *INO* in the central region was also reported in *sup-5* mutants (Villanueva et al., 1999). In *sup-5* mutants, the initial onset of *INO* expression was unaltered (Fig. 3G) but the subsequent expression of *INO* is similar to that seen in *nzz-2 ats* double mutants (compare Fig. 3H and J).

Expression of *BEL1* and *ANT* during ovule development in various mutants

It has been suggested previously that the expression of *INO* may involve a positive auto-regulatory loop (Villanueva et al., 1999). Our analysis of *INO* expression in *ino-2* also supports this hypothesis. Since *ANT* and *BEL1* are required for *INO*

expression, we asked whether this loop includes *ANT* or *BEL1* by analysing their expression in *ino* mutants. The expression of *BEL1* is similar in wild type and in *ino* suggesting that the auto-regulatory loop does not involve *BEL1* (Fig. 4A-D). Contrary to this, the expression of *ANT* shows variations from the wild-type expression pattern. While the expression of *ANT* is unaltered in other parts of the ovule, its expression in the outer integument shows subtle differences (Fig. 4E-H). In wild type, *ANT* is expressed in all the cells of the outer integument during its initiation. In *ino-2* mutants, the cells that would normally have given rise to the outer integument do not show strong expression of *ANT* (Fig. 4H), suggesting the involvement of *ANT* in the auto-regulatory loop of *INO*. We analysed whether the central regions in *ant* and *bel1* mutants retain their identity using *BEL1* and *ANT* respectively, as markers for central region. *BEL1* and *ANT* stripes can be seen in *ant-72F5* and *bel1-1460* mutants, respectively, suggesting that the central region retains the chalazal identity in these mutants (Fig. 4O,P). We have previously reported that in *nzz* mutants, a strong spot of epidermal *ANT* expression can be detected in cells that will give rise to the outer integument, and that the strong *NZZ* expression is reduced at the site where outer integument should be located in *ino* mutants (Balasubramanian and Schneitz, 2000) (Fig. 4M,N arrows). If the *ANT* spot in *nzz* is a result of the absence of negative regulation by *NZZ,* via the positive auto-regulatory loop of *INO*, then this spot should not be present in *nzz ino* double mutants. Therefore, we analysed *ANT* expression in *nzz-2 ino-2* double mutants. *ANT* expression is found throughout the ovule primordia at about stage 1-II (Fig. 4I). Around stage 2-I, *ANT* expression starts to disappear from the proximal region, but can still be observed in the distal region (Fig. 4J). Later in development, when an enlarged primordia is clearly visible, *ANT* expression can still be observed in the distal two thirds, similar to that seen in *nzz-2* (Fig. 4K,L). The distal region (Fig. 4La) and the growing nucellus (Fig. 4Lb) show *ANT* expression even around stage 2-III and later, in *nzz-2 ino-2* double mutants. As predicted, the strong spot of *ANT* expression, which is normally observed in *nzz-2* mutants, could not be detected in *nzz-2 ino-2* (Fig. 4K,L). Monitoring *ANT* expression at regular intervals throughout the colour detection period ruled out the possibility that we may not be seeing the difference in the levels of *ANT* expression at the 'spot' because of an overall strong signal. The distal expression of *ANT* is also consistent with our proposed model for PD pattern formation (see Discussion) (Balasubramanian and Schneitz, 2000).

DISCUSSION

Expression of *INO* requires *BEL1*

INO is expressed in the cells that give rise to the outer integument, before its initiation (Balasubramanian and Schneitz, 2000; Villanueva et al., 1999) (Fig. 2B). Genetically, *bel1* is epistatic to *ino* and *BEL1* has been suggested to be a negative regulator of *INO* in the chalaza (Villanueva et al., 1999). This conclusion is based on an observed ectopic *INO* expression in *bel1-1* at stage 4-I during ovule development (Villanueva et al., 1999). Since *INO* expression begins around stage 2-I, we analysed its expression in *bel1-1460* during all stages of development. We could not detect *INO* expression in

the mutant at this stage, which is in accordance with our previous analysis (Balasubramanian and Schneitz, 2000). However, we could not detect *INO* expression in *bel1* mutants even at later stages. Therefore, our results suggest that *BEL1* is a positive regulator of *INO*. If *BEL1* is a negative regulator of *INO*, then one would expect strong ectopic expression of *INO* in *nzz bel1* double mutants, since our analysis indicates that *NZZ* is a negative regulator of *INO*. Alternatively, if *BEL1* is a positive regulator, then *INO* expression should not be detected in *nzz bel1* double mutants. In *nzz bel1* double mutants, *INO* expression could not be detected, corroborating the need of *BEL1* for *INO* expression (S. B. and K. S., unpublished observations). The absence of *INO* expression in *bel1-1460* suggests that *INO* expression requires *BEL1* function. How does *BEL1* regulate *INO* expression? There are two possibilities. *BEL1* might directly regulate *INO* expression. Alternatively, *BEL1* might indirectly affect *INO* expression through its earlier role in chalazal specification. Our results do not distinguish between the two possibilities. However, since the identity of the central region does not seem to be changed in *bel1-1460* single mutants, as indicated by the expression pattern of *ANT* (Fig. 4O) as well as *BEL1* in *bel1-1460* (data not shown), this may not be due to a defect in chalazal identity. Instead, this may be due to a specific role of *BEL1* in integument development. The wild-type expression of *BEL1* in the developing integuments also supports this hypothesis (Reiser et al., 1995). We conclude that *BEL1* is a positive regulator of *INO* and is essential for its expression.

NZZ and *ANT* are temporal regulators of *INO* expression

Our analysis of the expression pattern of *INO* in *ant* and *nzz* indicates that the proper temporal expression of *INO* requires co-ordination of *ANT* and *NZZ* activities. *nzz* mutants show early initiation of the outer integument, which is preceded by the expression of *INO*, suggesting that *NZZ* is a negative temporal regulator of *INO* expression. In contrast to this, *ant* mutants show late onset of *INO* expression (Fig. 2H), suggesting that *ANT* is a positive temporal regulator of *INO* expression. *ANT* and *NZZ* act antagonistically in all regions of the ovule (Balasubramanian and Schneitz, 2000). The presence of *INO*, at later stages, in the strong *ant-72F5* mutant suggests that *ANT* is not absolutely required to turn on *INO*, rather *ANT* may be needed for the proper timing of *INO* expression. Taken together, these data suggest that the proper temporal expression of *INO* requires co-ordination of *NZZ* and *ANT* activities. Our analyses suggest a possible increase in the level of *INO* expression in *nzz* mutants. The antagonistic interaction of *NZZ* and *ANT* also leaves open the possibility that the expression of *INO* might equally well depend on the relative levels of *ANT* and *NZZ* activity. This negative regulation of *INO* by *NZZ* could be an essential temporal mechanism to couple PD patterning with Ad-Ab patterning (see below)

NZZ and *ATS* redundantly regulate *INO* expression and play a role in the maintenance of the adaxial-abaxial polarity

If *ANT* and *NZZ* regulate the temporal expression of *INO*, what regulates its spatial expression*?* Previously several loci were reported as negative regulators of *INO* expression (Villanueva et al., 1999). As we have shown above, *ANT* and *BEL1* are

positive regulators of *INO* expression. Contrary to this, *NZZ* acts as a negative regulator of *INO*, not only in a temporal manner but also in a spatial manner. Our analysis shows that *NZZ* and *ATS* redundantly negatively regulate *INO* expression in the adaxial chalaza (Fig. 3I-L). Any one of these loci is sufficient to restrict *INO* expression to the abaxial side since *ats* or *nzz* single mutants show normal abaxially located *INO* expression. How do *NZZ* and *ATS* regulate *INO* expression? We suggest at least two possibilities. First, *NZZ* and *ATS* are general negative regulators of *INO* expression and this inhibition is specifically overcome in the abaxial epidermis with the help of other factors such as *ANT*. The broad expression pattern of *NZZ* in the ovules (Balasubramanian and Schneitz, 2000; Schiefthaler et al., 1999) and the expression pattern of *ANT* in *nzz* mutants support such a hypothesis. The absence of the *ANT* 'spot' in *nzz ino* double mutants also supports this hypothesis. In *nzz ats*, the negative regulation by *NZZ* and *ATS* is absent and *INO* is expressed throughout the central region. Alternatively, *NZZ* and *ATS* might play a role only in the negative regulation of *INO* expression in the adaxial region. Is *NZZ* and *ATS* function required for initiation of the Ad-Ab axis or its maintenance? From our analysis it is clear that *NZZ* and *ATS* are definitely required for its maintenance. However, the weak misexpression of *INO* even in stage 2-I ovules of *nzz ats* double mutants suggests that they may play a role in the initiation of this axis as well. Why then is this misexpression weak at initial stages? It is possible that the *ats* allele available could be a weak allele. Since the molecular nature of *ATS* is not known, the nature of the *ats* allele is not clear.

NZZ, *ATS*, *INO* and *SUP* play a role in the asymmetric growth of the outer integument

INO and *SUP* have been previously reported to mediate the asymmetric growth of the outer integument (Gaiser et al., 1995; Schneitz et al., 1997; Villanueva et al., 1999). Our analysis of *nzz ats* double mutants indicates that *NZZ* and *ATS* are also required for the control of the asymmetric growth of the outer integument. Spatially, the ovules of *nzz ats* double mutants show no alterations in the initiation of the outer integument along the adaxial-abaxial axis. This indicates that *NZZ* and *ATS* are likely to play a role in the asymmetric growth of the outer integument after its initiation. Nevertheless, the altered expression pattern of *INO* at the initial stages itself in *nzz ats* (Fig. 3Ib) suggests that *NZZ* and *ATS* might also play a role in specifying this axis (see above). Since the single mutants of *nzz* and *ats* do not show any alterations in the asymmetric growth, we conclude that *NZZ* and *ATS* redundantly regulate the asymmetric growth of the outer integument.

SUP, *NZZ* and *ATS* are required for the maintenance of the adaxial-abaxial polarity during ovule development

SUP is another locus that regulates *INO* expression in the adaxial region of the chalaza. *SUP* has been suggested to be a negative regulator of *INO* expression (Villanueva et al., 1999). Our analysis of *INO* expression in *sup* mutants corroborates this hypothesis. The broader expression pattern of *INO* in *sup* mutants around stage 2-III is similar to that observed in *nzz ats* double mutants. How then does *SUP* relate to *NZZ* and *ATS*? Currently this remains an open question. However, we suggest

Table 1. A summary of the observed expression patterns of *INO* and *ANT*

Genotype	Observations	Interpretations
Expression summary for *INO*		
Wild-type L*er*	Initiation and expression at the abaxial epidermis of the proximal chalaza	
ino-2	Undetectable at any stage during development	Possible auto-regulation
bel1-1460	Undetectable at any stage during development	*BEL1* needed for *INO* expression
nzz-2	Early onset, can be detected at about stage 1-II	*NZZ* and *ANT* needed for the temporal regulation of *INO* expression
ant-72F5	Late onset, can be detected only by stage 4-V	
ats	Similar to wild type	*NZZ* and *ATS* redundantly regulate the spatial expression of *INO*
nzz-2 ats	Ectopic expression in chalaza starting from stage 1-II	
sup-5	Similar to *nzz ats*	*SUP* needed for spatial regulation of *INO*
Expression summary for *ANT*		
Wild-type L*er*	Chalaza, developing integuments	
nzz-2	Distally extended with a strong 'spot' at the site of outer integument initiation	*ANT* is involved in the auto-regulation of *INO*
ino-2	Similar to wild type with a gap of expression at the site of outer integument initiation	
nzz-2 ino-2	Similar to *nzz-2* but without the strong 'spot'	

at least three possibilities. First, *NZZ* and *ATS* function redundantly upstream of *SUP*. Second, *SUP* functions upstream of *NZZ* and *ATS*. Third, *NZZ*, *ATS* and *SUP* function at the same step of the cascade, in which case, *SUP* would be the central player. A genetic analysis of *nzz sup* double mutants did not allow us to discriminate between these possibilities as these mutants show *sup*-like ovules that lack the nucellus and thus exhibit an additive phenotype (data not shown). The observation that at least in some instances the *INO* expression is altered even at initial stages in *nzz ats* double mutants, suggests that *NZZ* and *ATS* might function earlier than *SUP*.

Outgrowth of the outer integument might require proper juxtaposition of the adaxial-abaxial signals

Why do the *ino* mutants produce ovules that lack the outer integument? It has been suggested that in *ino* mutants adaxialisation of the abaxial side of the outer integument leads to minimal or no outgrowth (Villanueva et al., 1999). Based on our analysis of wild-type *INO* expression, we propose a hypothesis to explain why adaxialisation should stop the outgrowth? Genetically *ino* is epistatic to *sup, nzz* and *ats* with respect to the outer integument, which fits well with the hypothesis that *INO* may be required for the initial outgrowth of the outer integument. As we have shown above, the tip cell, which usually enlarges and initiates the outgrowth, does not express *INO*. Then why should the absence of *INO* prevent the outgrowth? It has been suggested that juxtaposition of adaxial and abaxial signals may be needed for the outgrowth of lateral organs, similar to the requirement of dorsal and ventral signals during lateral appendage development in animals (Bowman, 2000b; Waites and Hudson, 1995). It is possible that in *ino* mutants such a juxtaposition of the adaxial and abaxial signals within the outer integument is not achieved because of the absence of *INO*. This fits well with the observation that *INO* expression is observed in only one cell layer of the outer integument and the absence of *INO* expression from the tip cell that enlarges to give rise to the outer integument. We suggest that the outgrowth of the outer integument requires proper juxtaposition of the adaxial and abaxial signals within the outer integument itself, which could be the reason why *ino* mutants lack the outer integument.

A model for outer integument development and regulation of *INO* expression

Our results are summarised in Table 1 and we propose a model that attempts to explain the genetic regulation of adaxial-abaxial pattern formation and outer integument development during ovule development (Fig. 5). *NZZ* plays a central role in different aspects of ovule development and it is required repeatedly during various stages of ovule development. We have previously proposed that *NZZ* and *BEL1* redundantly specify the chalaza (Balasubramanian and Schneitz, 2000). Furthermore, with respect to the integument development pathway, *NZZ* functions downstream of *ANT* and *INO* (Balasubramanian and Schneitz, 2000). In the model that we propose here, we suggest that initiation of the outer integument and Ad-Ab polarity establishment in the ovule occur after specification of the chalaza. This is achieved at least in part through the co-ordination of activities of *ANT, BEL1, NZZ, ATS* and *SUP*. The exact positioning of *INO* expression in the abaxial epidermis of the proximal chalaza may require other factors as well. Once *INO* is turned on, maintenance of its expression goes through an auto-regulatory loop that includes *ANT*. Subsequent *ANT* expression has a positive feedback on *INO* as well as *NZZ*. How does *INO* regulate *NZZ* expression? If *INO* regulates *ANT* via *NZZ*, then one would not detect increased levels (spot) of *ANT* in *nzz* mutants. Therefore, it is likely that the positive feedback regulation of *ANT* by *INO* is separate from the negative feedback of *NZZ* on *ANT* (Fig. 5B), though these two regulations are inter-connected. Thus, the positive feedback of *INO* on *ANT* leads to a positive regulation of *NZZ* by *ANT*. *NZZ* in turn has a negative feedback on *ANT*. In the adaxial side, *NZZ* redundantly regulates *INO* expression with *ATS*. This could be mediated via *SUP* or could be exerted independently (see above). If this model is true, then what would happen in a *nzz* mutant? In the absence of *NZZ*, *INO* is turned on precociously. Once *INO* is turned on, it leads to a positive feedback on *ANT* expression and consequently an increase in *INO* expression as well. This would also lead to an increase in *NZZ* expression in the outer integument. Since the NZZ protein is nonfunctional in *nzz-2*, this will lead to increased levels of *ANT* and *INO*. The predictions of this model hold true at least for *ANT* expression in *nzz-2*. *nzz-2* mutants

show an increased expression of *ANT* in the cells that give rise to the outer integument. This model is also supported by the fact that *INO* expression can be detected more easily in *nzz-2* than in wild type. Furthermore, this model also supports the absence of the *ANT* 'spot' in *nzz ino* double mutants.

Orchestration of proximal-distal and adaxial-abaxial pattern formation and growth

Our analysis indicates that *NZZ* links several aspects of ovule development. We have previously suggested that Ad-Ab and PD patterning are intimately coupled and that *INO* functions in a non-cell autonomous way (Balasubramanian and Schneitz, 2000). In this study, we show that *NZZ* and *ATS,* play a redundant role in patterning the adaxial-abaxial axis. Our results also suggests that the levels of *ANT* and *NZZ* are crucial for the proper growth and development of the ovule. From our present analysis, we suggest that the precocious expression of *INO* in *nzz*, which is normally the first event that molecularly marks the adaxial-abaxial axis, interferes with proximal-distal pattern formation in the primordium resulting in the absence of a nucellus and the presence of a longer funiculus. This may be, for example, analogous to the situation in the vertebrate limb, where alterations in the establishment of the anterior-posterior axis hinders proximal-distal patterning (Capdevila and Belmonte, 2001). Thus, by negatively regulating *INO* expression in a temporal manner *NZZ* makes sure that the onset of the Ad-Ab axis occurs at the correct time. Thereby, *NZZ* appears to co-ordinate pattern formation along both axes. We propose that *NZZ,* through its interactions with *BEL1,* a patterning gene involved in proximal-distal pattern formation, *INO,* a gene involved in adaxial-abaxial pattern formation and *ANT,* a gene that exerts growth control, links all these distinct processes with the help of genes such as *ATS* and *SUP*.

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