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 \textit{bell} mutants show abnormal outgrowths in place of integuments (Modrusan et al., 1994a; Ray et al., 1994; Reiser et al., 1995; Schneitz et al., 1997). \textit{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 \textit{BEL1} protein, the expression pattern of \textit{BEL1} and the \textit{bell} phenotype make \textit{BEL1} an excellent candidate for patterning the PD axis. Recently we have reported the genetic analysis of \textit{NOZZLE (NZZ)} and shown that \textit{NZZ} functions redundantly with \textit{BEL1} in specifying the chalaza (Balasubramanian and Schneitz, 2000). In the ovules of \textit{nzz \textit{bell} 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. \textit{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). \textit{NZZ} shows an antagonistic genetic interaction with \textit{AINTEGUMENTA (ANT)}, which encodes an AP2 domain-containing transcription factor (Elliott et al., 1996; Klucher et al., 1996). \textit{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 \textit{nzz}, we proposed that \textit{NZZ}, through its interactions with \textit{BEL1} and \textit{ANT}, couples PD pattern formation and growth during ovule development in \textit{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 \textit{phantastica (phan)} of \textit{Antirrhinum}, \textit{leafbladeless (lbl1)} of maize, \textit{laml} of \textit{Nicotiana} and \textit{argonaute (ago1)}, \textit{pinhead/zwille (pnh/zll)}, \textit{phabulosa (phb)} and \textit{phavoluta (phv)} of \textit{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 Marcorigiano, 1998; Timmermans et al., 1998; Waite and Hudson, 1995). Contrary to this, the members of the \textit{YABBY} gene family, which encode transcription factors, and the \textit{KANADI} genes, which also encode transcription factors, promote abaxial cell fate (Bowman, 2000а; Eshed et al., 1999; Golz and Hudson, 1999; Kerstetter et al., 2001; Siegfried et al., 1999). The members of the \textit{YABBY} gene family are expressed in a polar manner at the abaxial side of lateral organs. \textit{KANADI} genes redundantly promote abaxial cell fate possibly by negative regulation of the \textit{PHB/PHV} mediated adaxial signaling. When \textit{KANADI} function is compromised, adaxialised organs are formed (Eshed et al., 2001).

\textit{INNER NO OUTER (INO)}, a member of the \textit{YABBY} family plays a vital role in Ad-Ab pattern formation during ovule development (Villanueva et al., 1999). \textit{ino} mutants exhibit ovules that lack the outer integument (Baker et al., 1997; Schneitz et al., 1997) and \textit{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. \textit{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 \textit{sup} mutants, the outer integument initiates properly, but grows equally on both adaxial and abaxial side suggesting that \textit{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 \textit{INO} regulated in a spatial and temporal manner to ensure that such a co-ordination is achieved? Here we report the expression patterns of \textit{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 \textit{BEL1}, \textit{ANT} and \textit{NZZ} activities is required for the onset and temporal expression of \textit{INO}. We present evidence that \textit{NZZ} and \textit{ATS} spatially restrict the expression of \textit{INO} to the abaxial epiderm. We further report the expression patterns of \textit{ANT} in \textit{ino} and \textit{nzz} \textit{ino} double mutants and show that the positive auto-regulatory control of \textit{INO} expression (Villanueva et al., 1999) involves \textit{ANT}. We propose a model that summarises our findings and explains how Ad-Ab patterning and outer integument development occurs during ovule development in \textit{Arabidopsis}. Our analysis indicates \textit{NZZ} as a molecular link that orchestrates pattern formation along PD and Ad-Ab axis during ovule development in \textit{Arabidopsis}.

**MATERIALS AND METHODS**

**Plant growth and mutant alleles**

Plants were grown as described previously (Balasubramanian and Schneitz, 2000; Schneitz et al., 1997) and \textit{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: \textit{nzz-2, ant-72F5, bel1-1460, ats, ino-2 and sup-5}. All these mutant alleles have been described previously. \textit{nzz-2} is a putative null (Schiefthaler et al., 1999). \textit{ant-72F5, bel1-1460} show a strong phenotypes comparable to null alleles of \textit{bell} and \textit{ant} (Schneitz et al., 1997). The molecular nature of the single \textit{ats} allele is unknown since \textit{ATS} has not yet been cloned (Léon-Kloosterziel et al., 1994). \textit{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). \textit{sup-5} shows the strong ovule phenotype and has been described previously (Gaiser et al., 1995). \textit{nzz-2} \textit{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 \textit{INO, BEL1} and \textit{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 integment is initiated earlier than the inner integment (Fig. 1N) in nzz-2 ats double mutants. After initiation, the outer integment 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 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 bell-1-1460 (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 bell-1-1460 (M-O). Arrows in L indicate site of outer integument initiation in wild type. Arrows in O denote early chalazal bulges in bell-1-1460. Sense probe gave no signals above background (P). Scale bars: 20 μm.

Expression patterns of INO in wild type, ant-72F5, ino-2 and bell-1-1460

Mutations in the INO locus lead to lack of the outer integument (Baker et al., 1997; Schnitz 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 bell-1-1460 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 bell-1-1460

Since ant and bell 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 bell-1-1460. 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
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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. 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/II-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), 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.

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 prerequisite 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. Inats 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. 3Aa). Sometimes, it was more obvious, with INO expression detected throughout the central region similar to a BEL1 or ANT stripe (Fig. 3Ab). 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. 3b) 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
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. NZZ redundantly regulates the spatial expression of INO and ANT (Balasubramanian and Schneitz, 2000). In the model that we propose here, ANT is involved in the auto-regulation of INO. 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

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

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