INNER NO OUTER regulates abaxial–adaxial patterning in *Arabidopsis* ovules

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The *Arabidopsis* *INNER NO OUTER* (*INO*) gene is essential for formation and asymmetric growth of the ovule outer integument. *INO* encodes a member of the newly described YABBY family of putative transcription factors that contain apparent Cys₂–Cys₂ zinc-finger domains and regions of similarity to the high mobility group (HMG) transcription factors. In wild-type plants, *INO* is expressed specifically on one side of the central region of each ovule primordium in the cells that give rise to the outer integument. Alterations in the *INO* expression pattern in mutant backgrounds implicate *INO* as a positive regulator of its own expression, and *ANT*, *HLL*, *BEL1*, and *SUP* as direct or indirect negative regulators that help to establish the spatial pattern of *INO* expression. We hypothesize that *INO* is necessary for polarity determination in the central part of the ovule. Maintenance of polarity in other parts of *ino* ovules indicates the existence of additional regulators and provides further evidence that the ovule is a compound structure.

[Key Words: Polarity; transcription factor; YABBY; *INO*; plant development; integument]

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Consisting of only four morphological parts, the *Arabidopsis* ovule is a relatively simple structure that lends itself to the study of genetic regulation of pattern formation and organogenesis in plants (for review, see Gasser and Robinson-Beers 1993; Reiser and Fischer 1993; Angenent and Colombo 1996; Gasser et al. 1998; Schneitz et al. 1998b). Although initiating as a radially symmetrical primordium, the developing ovule subsequently exhibits differences between the side toward the apex of the carpel (adaxial) and the side toward the base of the carpel (abaxial)—resulting in a bilaterally symmetrical structure. Asymmetric development is most apparent in the outer integument (one of two sheathing structures) and in the funiculus (supporting stalk). The outer integument grows extensively only on the abaxial side of the ovule, and the funiculus curves in the abaxial direction. Two genetic loci involved in asymmetric development of the outer integument have been described. In addition to effects on flower development (Schultz et al. 1991; Bowman et al. 1992), the *SUPERMAN* (*SUP*) gene is essential for suppressing adaxial growth of the outer integument (Gaiser et al. 1995), and mutations in this gene lead to nearly equal outer integument growth on both sides of the ovule. In contrast, *inner no outer* (*ino*) mutations can lead to an absence of outer integument

growth on both sides of the ovule primordium, implicating *INO* as a positive regulator of integument growth or a determinant of polarity (Gaiser et al. 1995; Baker et al. 1997; Schneitz et al. 1997).

In angiosperms, bilateral symmetry is also a common characteristic of leaves, floral organs, and often whole flowers. In these structures, bilateral symmetry results from significant differences in development between the adaxial (toward the shoot apex) and abaxial (away from the shoot apex) sides. Recently, several genes involved in establishing these abaxial–adaxial patterns have been identified. The *cycloidea* (*cyc*) and *dichotoma* (*dich*) genes of *Antirrhinum majus* are expressed on the adaxial sides of flowers, where they specify adaxial floral development (Luo et al. 1996). In contrast, *phantastica* (*phan*), which appears to be essential for identity of the adaxial leaf surface in this same species, is expressed throughout the leaf (Waites et al. 1998), and must, therefore, require additional asymmetrically distributed factors for its activity.

In *Arabidopsis thaliana*, vegetative structures of the *phabulosa-1d* mutant are radially symmetrical, apparently as a result of adaxialization, and *PHABULOSA* may be one determinant of abaxial cell fate in this species (McConnell and Barton 1998). A recently described family of *Arabidopsis* genes, the YABBY genes, encoding putative transcription factors (Bowman and Smyth 1999; Kumaran et al. 1999; Sawa et al. 1999; Siegfried et al. 1999), participate in determination of abaxial identity in a variety of organs. Three members of this family, *FILAMENTOUS FLOWER* (*FIL*), *YABBY2* (*YAB2*), and *YAB3*,

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are expressed exclusively in the abaxial sides of cotyledons, leaves, and floral organs (Sawa et al. 1999; Siegfried et al. 1999). *FIL* and *YAB3* were shown to be determinants of abaxial identity in these organs (Sawa et al. 1999; Siegfried et al. 1999). *CRABS CLAW* (*CRC*), another member of this family, has roles in nectary development and carpel architecture (Bowman and Smyth 1999). *CRC* is expressed in nectaries and on the abaxial (external) face of carpels. Its role in abaxial development became apparent in the presence of second-site-enhancer mutations that cause carpels of *crc* mutants to exhibit partial adaxialization (Eshed et al. 1999).

Herein we describe the isolation and characterization of the *Arabidopsis INO* gene. We show that *INO* is a member of the YABBY gene family, and that, like the other family members, its asymmetric pattern of expression is closely associated with asymmetric development. Changes in the accumulation patterns of *INO* mRNA in different ovule mutant backgrounds identify several putative regulators of *INO* expression. The new data enable the formulation of models for the roles of both *INO* and *SUP* in regulation of polar development in ovules.

Results

Phenotypic effects of *ino* mutations

In wild-type plants, ovule primordia arise from the placental regions on the interior surfaces of the carpels as radially symmetrical finger-like projections. As the primordia grow, the funiculi bend toward the septum and

the base of the carpel (the abaxial direction; Fig. 1A,B). The inner integument is initiated from a symmetrical ring of cells encircling the central part of the ovule primordium, whereas the outer integument initiates from cells on the abaxial side of the ovule primordium (Fig. 1A). The zone of integument initiation (the chalaza) separates the distal region (nucellus) from the proximal region (funiculus) of the developing ovule. The inner integument continues to grow symmetrically (Fig. 1B). The outer integument exhibits marked asymmetric growth throughout its development, growing extensively on the abaxial side, and only to a very limited extent on the adaxial side (Fig. 1B). The resulting hood-shaped structure covers both the inner integument and the nucellus (Fig. 1C).

As described previously (Baker et al. 1997; Schneitz et al. 1997), in strong *ino* alleles (i.e., *ino-1* and *ino-2*) the outer integument fails to initiate on the abaxial side of the ovule, and subsequent outer integument development is not observed (Fig. 1D–F). However, a protuberance appears on the adaxial side of the ovule and undergoes limited expansion (Fig. 1D–F). In a newly identified weaker allele, *ino-4*, the outer integument initiates in the correct location on the abaxial side of an ovule primordium (Fig. 1G). However, subsequent growth of the outer integument is reduced as compared with wild type, and the outer integument fails to completely enclose the inner integument and nucellus (Fig. 1H–J). The adaxial protuberance observed in *ino-1* and *ino-2* was also observed in *ino-4* but was partially covered by the reduced outer integument (Fig. 1I,J). *ino* mutations caused no vis-

Figure 1. Scanning electron micrographs of wild-type and *ino* ovules. In all panels, the base of the ovary (and therefore, the abaxial side of the ovules) is at left. Bars, 10 μ m. (A) Wild-type ovules at stage 2–III (stages according to Schneitz et al. 1995). The nucellus, funiculus, and inner and outer integument primordia are visible. The outer integument initiates on the abaxial side of the wild-type ovule. (B) Wild-type ovules at stage 3–I. The outer integument encloses most of the inner integument and nucellus. (C) Wild-type ovules at anthesis (stage 4–I). The outer integument completely covers the inner integument and nucellus. (D) *ino-1* ovules at stage 2–III. The outer integument on the abaxial side of the ovule is absent. A protuberance (arrowheads) forms on the adaxial side of each *ino-1* ovule. (E) *ino-1* ovules at stage 3–I. (F) *ino-1* ovules at stage 4–I. The inner integument completely covers the nucellus and the adaxial protuberance (arrowheads) is enlarged. (G) *ino-4* ovules at stage 2–III. Initiation of the outer integuments on the abaxial sides is apparent (arrows). (H) *ino-4* ovules at stage 3–I. The outer integuments are reduced relative to wild type. The adaxial protuberance is visible on some ovules (arrowhead). (I,J) *ino-4* ovules at stage 4–I. The outer integuments are reduced relative to wild type. The adaxial protuberance (arrowheads) protrudes between the inner and outer integuments. (f) funiculus; (ii) inner integument (primordium); (n) nucellus; (oi) outer integument (primordium).

ible alterations in other aspects of ovule, flower, or vegetative development (Fig. 1; Baker et al. 1997; Schneitz et al. 1997).

Positional cloning of *INO*

Because all available *ino* alleles derived from chemically mutagenized populations, we used a positional-cloning approach to isolate the *INO* gene. The physical position of *INO* was refined to within a region containing four putative genes (numbered 5–8) on two overlapping cosmid clones (cJMV41 and cJMV43). The sequences of genes 7 and 8 amplified from the genomes of mutant lines revealed putative mutations only in gene 7 for all three alleles (Fig. 2A,B). The independently isolated *ino-1* and *ino-2* mutants had a G-to-A transition near the splice acceptor site of intron 5 (of six total introns), and

a similar transition was found near the intron 4 splice acceptor site in *ino-4* (Fig. 2A,B). Gene 7 RT-PCR products were generated and sequenced from RNA of both wild-type strains and the three *ino* alleles. When compared with wild type, cDNAs from *ino-1* and *ino-2* included 11 additional nucleotides due to utilization of an aberrant splice-acceptor site created by the mutation (Figure 3B). This resulted in a frameshift in translation of the carboxy-terminal region of the predicted protein, replacing the last 59 amino acids with 75 essentially random residues. The mutation in the weak *ino-4* allele formed a new splice-acceptor site for intron 4, causing the inclusion of 15 additional nucleotides in the mRNA (Fig. 2B). These nucleotides translate into a five-amino-acid insertion, leaving the rest of the protein in frame. In all cases, the unambiguous sequence obtained directly from the RT-PCR products indicated that essentially all of the mRNA in the mutants derived from use of the

Figure 2. *INO* DNA and protein sequences. (A) *INO* cDNA and predicted protein sequence (Co-3 ecotype). Coding region of cDNA is shown in uppercase and 5'- and 3'-untranslated regions are in lowercase. Nucleotide differences in the coding region of the Ler ecotype are shown above the cDNA sequence. Single and double underlines indicate the putative zinc finger and YABBY domains, respectively. Conserved cysteine residues in the putative zinc finger region are in boldface type. (▽) Intron locations, and introns containing the *ino-1*, *ino-2*, and *ino-4* mutations are indicated. (Asterisks) Stop codons. (B) Mutations lead to altered splicing. Wild-type genomic sequences of introns 5 and 4, with some flanking exon sequence, are illustrated at top of each panel. Wild-type exons and the amino acids they encode are shown in boldface and donor (GT) and acceptor (AG) intron splice sites are overlined. Vertical gray bars indicate G-to-A transitions identified in the mutant genomic sequences. Mutant cDNA sequences are illustrated below each genomic sequence. Mutant donor and acceptor sites are indicated by black underlines. Alterations in the protein sequence resulting from aberrant splicing in the mutants are indicated by gray underlines. (C) Alignment of conserved putative zinc finger (top) and YABBY (bottom) domains. Black boxes indicate identical and gray boxes indicate conserved residues between *INO* and other proteins. Broken lines indicate gaps in the sequences. The overline indicates predicted α -helical domains in *INO* (by the method of Chou et al. 1978); the underline indicates α helices in *SRY* (Werner et al. 1995).

Figure 3. Histochemical detection of in situ hybridization with an *INO* antisense RNA probe in wild-type ovules. In all panels, the base of the carpel and the abaxial side of the ovules, is at *left*. Bars, 10 μ m. (A,B) Stage 2-I ovule primordia (before emergence of integuments). (A) Longitudinal sections show *INO* mRNA at the site of origination of the outer integument on the abaxial sides of three ovule primordia (two pointing upward and one pointing downward). (B) Cross sections show that *INO* mRNA is confined to a semicircular collar of cells corresponding to the site of initiation of the outer integument. (C) Longitudinal section of ovules at stage 2-II. *INO* mRNA is in the abaxial layers of only the outer integument primordium. (f) Funiculus; (ii) inner integument primordium; (n) nucellus; (oi) outer integument primordium.

mutant splice junctions (data not shown). These data were consistent with gene 7 being *INO*.

Complementation of *ino-1* by transformation

Cosmid cJMV41, which contains gene 7, was used for in planta transformation. Homozygous *ino-1* plants containing cJMV41 displayed the *Ino*⁺ phenotype, demonstrating that this cosmid contained *INO*. A second construct made of the gene 7 cDNA flanked by ~2.3 kb of 5' and 2.0 kb of 3' gene 7 genomic DNA was transferred into plants segregating for *ino-1*. Five of six independent transformants homozygous for *ino-1* were *Ino*⁺, showing that the gene 7 cDNA transgene complements *ino-1*. Together, these data demonstrate that gene 7 is *INO*.

INO encodes a protein with zinc-finger and HMG-like domains

The Co-3 *INO* cDNA encodes a 231-amino-acid protein that includes a putative Cys₂-Cys₂ zinc-finger domain

near the amino terminus, and a high mobility group (HMG)-like domain (Landsman and Bustin 1993; Grosschedl et al. 1994; Baxevanis and Landsman 1995) near the carboxyl terminus (Fig. 2A). The presence of these two domains is characteristic of the recently described YABBY family of putative transcription factors (Bowman and Smyth 1999; Sawa et al. 1999; Siegfried et al. 1999). The HMG-like domain of these proteins, designated the YABBY domain (Bowman and Smyth 1999), has sequence similarity to DNA-binding domains of HMG transcription factors such as the human SRY protein (Fig. 2C; Werner et al. 1995). The HMG-like domain of SRY consists of three α -helices separated by turns, with the first two helices binding directly to DNA (Werner et al. 1995). The YABBY domains align with the first two helices of this motif and are predicted to form two α -helices separated by a turn (Fig. 2C; data not shown).

INO sequences outside of the two conserved domains show no special similarity in amino acid composition or length to the corresponding regions of other *Arabidopsis* YABBY proteins (Siegfried et al. 1999; data not shown). The region of *INO* between the putative zinc finger and YABBY domains is hydrophilic and acidic (21% glutamate plus aspartate), whereas in other YABBY proteins this region can be, for example, proline rich (YAB3; Siegfried et al. 1999), or serine rich (CRC; Bowman and Smyth 1999).

We note that a sequence difference in the Landsberg *erecta* (Ler) ecotype introduces a stop codon at the fourth codon of the conceptual translation (Fig. 2A). This indicates that in this ecotype (and possibly in Co-3 as well) translation must initiate at the second start codon, producing a protein that is six amino acids shorter at the amino terminus.

Expression pattern of *INO* in wild-type ovules

The spatial distribution of *INO* transcript accumulation was examined by in situ hybridization with an *INO* antisense probe (omitting the YABBY domain). *INO* mRNA was first detected in a group of ~15 epidermal cells on the abaxial half of each ovule primordium, prior to visible emergence of the integuments [stage 2-I of ovule development (Schneitz et al. 1995; Fig. 3A,B)]. These expressing cells correspond to the site of outer integument initiation (Fig. 3A,B). At stage 2-II/III, when outer and inner integument primordia are visible, *INO* mRNA was detected only in the outer integument on the abaxial side of the ovule primordium (Fig. 3C). At anthesis, *INO* mRNA was no longer present at detectable levels (data not shown). *INO* mRNA was not apparent in any other floral structures, although some putative signal was seen in all cells of globular, but not later stage embryos (data not shown). No hybridization was detected with the *INO* sense probe (data not shown).

INO expression in mutant backgrounds

We were unable to detect *INO* mRNA in ovules of the strong *ino-1* allele by in situ hybridization, indicating

that levels of accumulation in this mutant must be substantially lower than in wild type. *INO* mRNA was detected in the weaker *ino-4* allele in the same pattern observed in wild-type ovules (data not shown). The relatively weak signal in these hybridizations indicated that *INO* mRNA may be accumulating to lower levels in *ino-4* than in wild type.

In *huellenlos-1* (*hll-1*) mutant ovules, only rudimentary integumentary ridges emerge from the chalaza (Fig. 4A; Schneitz et al. 1998a). Development ceases shortly thereafter, and cells of the nucellus commonly degenerate. *INO* transcripts were observed both in the chalaza and in the nucellus of *hll-1* ovules (Fig. 4B). *INO* mRNA

Figure 4. Distribution of *INO* mRNA in mutant ovules. Scanning electron micrographs (A,C,E,G,I) and in situ hybridizations with an *INO* antisense RNA probe (B,D,F,H,J) of mutant ovules. In all panels, the base of the carpel and the adaxial side of the ovules is at left. Bars, 10 μ m. (A,B) Stage 4-I *hll-1* ovules. *INO* mRNA is detected in the integumentary ridge and nucellus but not in the funiculus. Note that the ridge is not evident in all ovules. (C,D) Stage 4-I *ant-4* ovules. *INO* mRNA is detected throughout the integumentary ridge. (E,F) Stage 4-I *bel1-1* ovules. *INO* mRNA is detected uniformly throughout the integument-like structure of *bel1* ovules. (G,H) Stage 4-I *sup-5* ovules. *INO* mRNA is detected on both sides of the base of the outer integument and in the funiculus. (I) Stage 4-I and (J) stage 2-III *ats* ovules. *ats* ovules have only a single integument (Leon-Kloosterziel et al. 1994). During early development of this structure, *ino* mRNA was detected only in the basal half of the abaxial side of the single integument primordium. (ir) Integumentary ridge; (n) nucellus; (f) funiculus; (il) integument-like structure; (i) single integument; (oi) outer integument.

was not specific to the abaxial side of the ovule, but was evenly distributed across the chalaza and nucellus.

In the strong *aintegumenta-4* (*ant-4*) mutant, in which the integuments are almost completely absent (Fig. 4C; Elliott et al. 1996; Klucher et al. 1996; Baker et al. 1997; Schneitz et al. 1998a), *INO* mRNA was detected only in the chalaza, in which it was evenly distributed along the abaxial–adaxial axis (Fig. 4D).

In *bel1-1* (*bel1-1*) ovules, a single integument-like structure emerges from the chalaza (Fig. 4E; Robinson-Beers et al. 1992). *INO* transcript was uniformly distributed throughout the integument-like structure, and also extended into the funiculus of *bel1-1* ovules (Fig. 4F).

In *sup* mutants, following apparently normal formation of an asymmetric outer integument primordium, the outer integument grows nearly equally on all sides of the ovule, resulting in a long tubular structure (Fig. 4G; Gaiser et al. 1995). In *sup-5* ovules, the zone of *INO* transcript accumulation extends across both sides of the outer integument and down into the funiculus (Fig. 4H).

In *aberrant testa shape* (*ats*) mutants, a single integumentary structure is observed in place of the two integuments (Fig. 4I; Leon-Kloosterziel et al. 1994). This single integument has properties of both inner and outer integuments, and has been hypothesized to result from fusion between these two structures (Leon-Kloosterziel et al. 1994). In *ats* ovules, the *INO* transcript is detected only in the proximal half of the single integumentary structure, but its distribution along the abaxial–adaxial axis is as in wild type (Fig. 4J).

Discussion

INO is a member of the YABBY gene family

The YABBY genes encode a newly defined class of putative plant transcription factors (Bowman and Smyth 1999; Kumaran et al. 1999; Sawa et al. 1999; Siegfried et al. 1999). The conserved Cys₂–Cys₂ putative zinc-binding and HMG-like domains of *INO* make it a member of the YABBY gene family. The patterns of mRNA accumulation of four other YABBY genes have been described (Bowman and Smyth 1999; Sawa et al. 1999; Siegfried et al. 1999), and *INO* is the only member that shows expression in ovules. *INO* is present in the recently released genomic survey sequence of BAC clone F26F24 and this sequence was referred to previously as *YABBY4* in Siegfried et al. (1999).

The demonstrated zinc-binding activity of FIL (Sawa et al. 1999) supports the hypothesis that the conserved Cys₂–Cys₂ domains of YABBY proteins represent zinc-binding structures. There is significant conservation of amino acid residues beside the cysteines among these domains in the different YABBY proteins (Fig. 2B; Bowman and Smyth 1999; Sawa et al. 1999; Siegfried et al. 1999). However, the absence of significant sequence similarity to previously described zinc finger domains indicates that the YABBY proteins may define a structurally distinct class of zinc-binding proteins. Regions outside of the zinc finger and YABBY domains exhibit

little or no sequence conservation among most members of the YABBY family, and may define functional specificity.

ino mutant effects

On the basis of the phenotypes of strong *ino* alleles, we had hypothesized previously that the protuberance forming on the adaxial side of *ino* ovules represented an abortive outer integument in an incorrect location (Baker et al. 1997). The presence of both an outer integument and the adaxial protuberance in the newly characterized *ino-4* mutant leads us to suggest other hypotheses. One alternative hypothesis is that the adaxial protuberance results from abnormal manifestation (due to the absence of an outer integument) of a normal process of ovule development. In wild-type *Arabidopsis* ovule development, following initiation of both integuments, there is subepidermal cell division on the adaxial side of the ovule in the region corresponding to the point of attachment of the two integuments (see, e.g., Fig. 2A of Robinson-Beers et al. 1992). We hypothesize that in the absence of outer integument development, this same cell division could initiate earlier and form the exposed abaxial protuberance in *ino-1*. Thus, it is possible that the only direct effect of a strong *ino* mutation is elimination of the outer integument. This is consistent with our observation that *INO* mRNA accumulates only in this structure. Although other possibilities remain, the phenotypic effects of severe *ino* mutations can be explained as primary and secondary results of absence of outer integuments.

The role of *INO* in outer integument development

The specific accumulation of *INO* mRNA in the cells giving rise to the outer integument suggest a simple model for the action of *INO* in which it acts as a general promoter of growth in the region in which it is expressed. The absence of *INO* activity in strong *ino* mutants would eliminate this influence, leading to a failure of the outer integuments to expand. This model is also consistent with our observation that in *sup* mutants, the near uniform expression of *INO* across the outer integument correlates with uniform growth of this structure on all sides of the *Sup*[−] ovule. Thus, the effects of *sup* mutations on ovule development may result only from changes in *INO* expression, consistent with *ino* being epistatic to *sup* in ovule development (Gaiser et al. 1995). *INO* cannot be sufficient to promote growth but must require other factors such as *ANT*, because in *ant* mutants there is no growth from the chalazal region despite significant *INO* expression (Fig. 4D). The need for other factors, which could be integument specific, could explain the lack of aberrant growth from the nucellus and funiculus in *hll*, *bel1*, and *sup* mutants in which *INO* is expressed in these structures (Fig. 4).

The roles of other YABBY genes in plant development suggest an alternative model for the role of *INO*. Express-

sion of *CRC*, *YAB2*, *YAB3*, and *FIL* has been shown to be on the abaxial sides of leaves and primary floral organs, and *CRC*, *YAB3*, and *FIL* have been shown to be important promoters of abaxial identity in these structures (Bowman and Smyth 1999; Eshed et al. 1999; Sawa et al. 1999; Siegfried et al. 1999). The outer integument normally grows extensively on the abaxial side of the ovule, and shows almost no growth on the adaxial side (Fig. 1). If *INO* is necessary for abaxial identity in the part of the chalaza giving rise to the outer integument, then its absence would lead to adaxialization of this region. Because limited growth is characteristic of the adaxial side of the outer integument, this would result in limited, or no outer integument growth on either side of the ovule primordium. The model can also explain the *sup* mutant phenotype, in which expansion of *INO* expression to the adaxial side of the ovule would lead to abaxialization of this surface and more symmetrical growth of the integument. Because this second model hypothesizes *INO* to be only a determinant of polarity in a developing integument, the model is consistent with the absence of phenotypic effects of ectopic *INO* expression observed in the nucellus and funiculus in *bel1*, *hll*, and *sup* ovules.

The apparent conservation of function of YABBY genes in polar identity determination leads us to favor the second model in which *INO* is a primary determinant of abaxial identity of a part of the chalaza including the outer integument. We note that our definition of abaxial side of an ovule is only by analogy to the abaxial side of leaves (Gaiser et al. 1995). Thus, our model hypothesizes only a conservation of YABBY function in polar identity, rather than in a specific side of a polar structure.

Regulation of *INO* expression

Changes in patterns of *INO* mRNA accumulation in mutant backgrounds indicate roles for *SUP*, *HLL*, *ANT*, and *BEL1* in processes regulating *INO* expression. *SUP* has been shown previously to have a cadastral function in specification of the inner boundary of expression of the *APETALA 3* (*AP3*) floral organ identity gene (Schultz et al. 1991; Bowman et al. 1992; Sakai et al. 1995). There are a number of striking parallels between the role of *SUP* in regulation of *AP3* and *INO*. For both genes, in *sup* mutants there is expansion of expression outside of the region of normal mRNA accumulation. Expression expands into the fourth floral whorl for *AP3* (Bowman et al. 1992), and to the adaxial side of the ovule for *INO* (Fig. 4H). For *AP3*, the expanded zone of expression can explain most effects of *sup* mutations on floral organ identity (Jack et al. 1994; Sakai et al. 1995). However, changes in *AP3* expression cannot account for *sup* effects on ovule development (Gaiser et al. 1995). As outlined above, we now show that the effects of *sup* mutations on ovule development may be completely mediated by the expansion of *INO* expression to the adaxial side of the ovule. We would therefore predict that ectopic expression of *INO* in ovules would lead to this same phenotype.

In both floral organ identity and ovule development, the cells in which gene expression is repressed by SUP are adjacent to the cells in which SUP mRNA accumulates. SUP mRNA accumulates in the third whorl of floral organs, but inhibits expression of AP3 in the fourth whorl by an apparently cell nonautonomous mechanism (Sakai et al. 1995). In the ovule, SUP mRNA was observed only in a small region on the adaxial side of the funiculus just below the site of outer integument initiation (Sakai et al. 1995). However, it is in the adjacent chalazal region that SUP appears to repress INO expression. Therefore, it is possible that SUP negatively regulates INO in ovules and AP3 in flowers by a common cell nonautonomous mechanism.

The expansion of the zone of INO mRNA accumulation in *hll*, *ant*, and *bel1* mutants (Fig. 4) implies that all three of these genes have roles in repressing INO expression in areas outside of the abaxial side of the chalaza. This is consistent with previous observations that all three mutations are epistatic to *ino* and apparently act earlier in ovule development (Baker et al. 1997; Gasser et al. 1998; Schneitz et al. 1998b). ANT and BEL1 are putative transcription factors (Reiser et al. 1995; Elliott et al. 1996; Klucher et al. 1996) and could directly regulate INO expression. However, neither protein could perform this function alone, because both are expressed throughout the chalaza (Reiser et al. 1995; Elliott et al. 1996; Klucher et al. 1996), including the region in which INO is normally expressed. They would have to act together with the products of other genes with more restricted or overlapping patterns of expression to suppress INO expression only on the adaxial side of the ovule primordium.

Expansion of the zone of INO expression could also be an indirect result of other effects of *hll*, *ant*, and *bel1* mutations. These three genes have been proposed previously as regulators of proximal-distal patterning or growth (Reiser et al. 1995; Elliott et al. 1996; Klucher et al. 1996; Schneitz et al. 1998a), and disruption in any of these early acting regulators could interrupt establishment of the developmental zones within the ovule. Thus, our results may indicate a linkage between proximal-distal and abaxial-adaxial patterning in ovules.

Our inability to detect INO mRNA in *ino-1* ovules by in situ hybridization indicates a dramatic reduction in mRNA levels in the mutant. Because the alteration in *ino-1* leads only to inclusion of 11 additional nucleotides in the INO transcript through a preferential splicing event, it is unlikely to have a significant negative effect on splicing efficiency or mRNA stability. However, interruption of the reading frame prior to the conserved tryptophan residue in the YABBY domain makes it likely that the mutant protein is inactive. The simplest explanation for the reduction of mRNA in the *ino-1* mutant would be for INO to be a positive transcriptional regulator of INO. Because INO appears to be a transcription factor, this regulation could be direct. Thus, following initial activation of INO expression, the presence of active INO protein could maintain expression in the initially activated cells. Such autoregulation has been ob-

served previously in maintenance of boundaries of expression for floral organ identity genes such as *APETALA3* and *PI* (Goto and Meyerowitz 1994; Jack et al. 1994).

Perspective

The evidence presented above suggests that INO is involved in polarity of only one small region of the ovule primordium, the anlage to the outer integument. This contrasts with other characterized YABBY genes that appear to regulate abaxial-adaxial development in entire leaves or primary floral organs (Bowman and Smyth 1999; Sawa et al. 1999; Siegfried et al. 1999). This may indicate diversification of the YABBY family through expansion or contraction of ancestral expression patterns, but is more likely additional evidence that the ovule is a compound structure. The fossil record indicates that the structures of an angiosperm ovule have been added sequentially in a number of events that may be separated by hundreds of millions of years (Stewart 1983; Gasser et al. 1998). The precursor to INO may have become incorporated into ovule development at the origin of the outer integument, or with the possibly separate origin of asymmetry of this structure. Analysis of patterns of expression of INO orthologs in ancestral species could differentiate between these hypotheses. Integumentary fusion has been proposed as one mechanism for the derived origin of unitegmic ovules in the angiosperms (Bouman 1984). In *ats* mutants, the pattern of INO mRNA accumulation in only the proximal part of the single integument (Fig. 4J) supports the hypothesis that this mutation results in fused integuments. Examination of the expression of INO orthologs in unitegmic angiosperms could test the hypothetical origin of their ovule morphologies. Further analysis of INO thus promises not only to illuminate the regulation of ovule ontogeny but may also help us to understand the process of ovule evolution.

Materials and methods

Plant material

Isolation of *ino-1* and *ino-2* in the Ler background was described previously (Baker et al. 1997; Schneitz et al. 1997). *ino-4* was isolated in the Columbia-3 (Co-3) background by similar methods, and was a kind gift of Imran Siddiqi (CCMB, Hyderabad, India). Mutants were backcrossed at least three times to wild-type plants of the parental ecotype prior to further analysis. Allelism was determined by genetic complementation tests (data not shown).

bel1-1 (Robinson-Beers et al. 1992), *ant-4* (Baker et al. 1997), *ats* (Leon-Kloosterziel et al. 1994), *hll-1* (Schneitz et al. 1998a), and *sup-5* (Gaiser et al. 1995) were described previously in the cited references.

Plants were grown as described previously (Kranz and Kirchheim 1987; Robinson-Beers et al. 1992).

Scanning electron microscopy

Samples were prepared as described previously (Robinson-Beers et al. 1992) and were examined with a Hitachi (Tokyo, Japan)

S3500N scanning electron microscope at an accelerating voltage of 5 or 10 kV. Images were acquired digitally and were edited for publication in Photoshop 4.0 (Adobe Systems, San Jose, CA).

Genetic and physical mapping

INO was mapped previously to chromosome 1 (Baker et al. 1997). More detailed mapping utilized DNA samples (Edwards et al. 1991) isolated from a total of 974 F₂ plants from a cross of *ino-1* (in the Ler background) to Co-3 wild-type plants. Informative recombinants were identified from this set by testing all samples for recombination between the cleaved amplified polymorphic sequence (CAPS) markers for the m235 (Hardtke and Berleth 1996) and *CAULIFLOWER* (*CAL*) (Kempin et al. 1995) loci, which flank *INO* and are separated by ~2.3 cM. The *CAL* CAPS marker was generated with gene-specific primers (a gift from Martin Yanofsky, University of California, San Diego) and digestion with *SacI* or *DpnII*. Genetic maps were determined with MapMaker 2.0 for Macintosh (Lander et al. 1987).

Further mapping utilized markers including g17286 (*HindIII* digest; Hauge et al. 1993), AthZFPG (Bell and Ecker 1994), and EST ve037 detecting two distinct but proximal loci (ve037E, *EcoRV* digestion, and ve037T, *TaqI* digestion), with these last two markers mapping 0.33 and 0.05 cM north and south of *ino*, respectively.

Bacterial artificial chromosome (BAC) T29E11 was identified by probing the TAMU-BAC library (Choi et al. 1995) with a ³²P-labeled ve037. T29E11 has a ~70-kb insert and contains both ve037 polymorphisms. T29E11 was partially digested with *Sau3A1* and DNA fragments from 17 to 25 kb were isolated by fractionation on a 10%–40% sucrose gradient and were subcloned into the *BamHI* site of the pOCA28 plant transformation cosmid vector (Olszewski et al. 1988). Cosmids were packaged in vitro (Stratagene, La Jolla, CA) and introduced into vcs257 and JM101 bacterial strains. Comparison of restriction endonuclease digestion patterns and hybridization analysis with isolated ³²P-labeled cosmid clones enabled the construction of a cosmid contig encompassing T29E11 (not shown). Additional RFLP markers were generated through hybridizations with the cosmids or cosmid subclones, allowing refinement of the position of *INO* to within two overlapping cosmids, cJMV41 and cJMV43.

For DNA sequence determination, cosmids cJMV41 and cJMV43 were partially digested with *Sau3A1*, fragments of ~1–8 kb were isolated by sucrose density gradient centrifugation (see above), and were subcloned into the *BamHI* site of pUC119 (Vieira and Messing 1987). Cosmid end-rescue was performed by self-ligation following *HindIII* or *EcoRI* digestion, followed by PCR with primers to the COS site and the ColE1 replicon of the pOCA28 vector (Olszewski et al. 1988) (*EcoRI* primer, GCCGC-TGTAAAGTGTTACGTTG; *HindIII* primer, GACGCAGG-TATCGTATTAATTG). PCR products were purified and sequenced directly. Putative genes in this region, numbered 1–9, were identified with NetPlantGene (<http://www.cbs.dtu.dk/NetPlantGene.html>, gene 5) or by alignment with cDNA sequences (other genes).

Isolation of *INO* cDNAs

An *Arabidopsis* (Ler) floral cDNA library (a gift of Detlef Weigel, Salk Institute, San Diego, CA) was screened with cJMV41 and cJMV43 insert DNA as probes. Two different cDNAs were isolated, one of which derived from *INO* (pJMV50). To determine the complete sequence of the 5' region of the *INO* mRNA, 5' rapid amplification of cDNA ends (RACE; Frohman 1990) was performed according to Harper et al. (1990), except for the

use of a gene specific primer complementary to bases 912–933 of the *INO* cDNA (Fig. 2A). PCR products were subcloned into pBluescript KS+ plasmid (Stratagene, La Jolla, CA) and transformed into JM101 cells.

To determine the sequence of *INO* mRNA in the mutant lines, first-strand cDNA was synthesized from total RNA isolated from wild-type Co-3 and the *ino* mutants. *INO* cDNA-specific primers corresponding to bases 32–51 and 912–933 in the *INO* cDNA (Fig. 2A) were used to amplify the *INO*-coding regions by PCR, and the products were sequenced directly with these same primers.

DNA sequence analysis

DNA was sequenced with Big Dye terminators (Perkin-Elmer Corp., Norwalk, CT) and the Prism-377-XL system (PE Applied Biosystems, Foster City, CA). Sequences were assembled with Sequencher 3.0 software (GeneCodes Corp., Ann Arbor, MI) and Genetics Computer Group software (v. 9.1, Genetics Computer Group, Madison, WI). Database searches were performed at the National Center for Biotechnology Information web site (<http://www.ncbi.nlm.nih.gov/>).

Complementation by transformation

Plasmid or cosmids were introduced into the ASE strain of *Agrobacterium tumefaciens* (Fraley et al. 1985) by triparental mating (Figurski and Helinski 1979), for in planta transformation of *Arabidopsis* (Bechtold and Pelletier 1998). cJMV41 was introduced into the Co-3 ecotype and transformants were selected on 50 µg/ml kanamycin and confirmed by gel blotting and hybridization with ³²P-labeled pOCA28 vector. One transformant was crossed with pollen from a homozygous *ino-1* (Ler) plant. Four of 16 F₂ plants were homozygous for the Ler alleles of the m235 and AthZFPG markers, which are closely linked to and flank *ino*, indicating that they were homozygous for *ino-1*. Two of these plants were phenotypically Ino⁺ (consistent with segregation of the transforming DNA), demonstrating that the transgene could complement the *ino-1* mutation.

The *INO* cDNA was inserted between the 5' (2.3 kb extending to the first *SaII* site) and 3' (2 kb extending to the first *EcoRI* site) flanking genomic regions of *INO*. This genomic::cDNA chimera was inserted into the pMLBART (a gift from Kim Richardson, Hort Research, Auckland, NZ) transformation vector (Gleave 1992), resulting in plasmid pRJM33. pRJM33 was transformed into plants segregating for the *ino-1* allele (in the Ler background) and the *INO* allele (in the Co-3 background). Transformants were selected for phosphinothricine (BASTA) resistance and verified by PCR reactions with gene-specific primers that amplify different sizes of fragments from the endogenous gene and the cDNA transgene. Transformants homozygous for the *ino-1* allele (in the Ler background) were identified as those homozygous for the Ler allele of the m235 CAPS marker (0.33 cM north of *ino*).

In situ hybridization

Sections of plant tissue 8–10 µm thick were prepared and hybridized as described previously (Vielle-Calzada et al. 1999) with the *INO* sense and antisense probes. Templates for *INO* probe preparation were constructed by subcloning a ~450-bp *BamHI*/*SacI* fragment of pJMV50 including sequence encoding the zinc finger domain, but not the YABBY domain, into pBluescript KS+ plasmid vector digested with these same enzymes. The resulting plasmid, pJMV86, was linearized for synthesis of sense and antisense probes by digestion with *SacI* or *XhoI*, respectively.

The 3' overhangs of *SacI* digestions were filled in with T4 DNA polymerase prior to probe synthesis. Digoxigenin-labeled probes were synthesized with the Genius RNA Labeling kit (Boehringer Mannheim Corp., Indianapolis, IN) according to the manufacturer's instructions. Sections were photographed on a Zeiss (Oberkochen, Germany) Axioplan microscope with differential interference contrast optics. Images were processed as described above.

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Note

Sequence data described in this paper have been submitted to GenBank under accession number AF195047.

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***INNER NO OUTER* regulates abaxial – adaxial patterning in *Arabidopsis* ovules**

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