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SUPERMAN attenuates positive *INNER NO OUTER* autoregulation to maintain polar development of *Arabidopsis* ovule outer integuments

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SUMMARY

The outer integument of *Arabidopsis* ovules exhibits marked polarity in its development, growing extensively from the abaxial side, but only to a very limited extent from the adaxial side of the ovule. Mutations in two genes affect this asymmetric growth. In strong *inner no outer* (*ino*) mutants outer integument growth is eliminated, whereas in *superman* (*sup*) mutants integument growth on the adaxial side is nearly equal to wild-type growth on the abaxial side. Through complementation and reporter gene analysis, a region of *INO* 5′-flanking sequences was identified that contains sufficient information for appropriate expression of *INO*. Using this *INO* promoter (P-INO) we show that INO acts as a positive regulator of transcription from P-INO, but is not sufficient for de novo initiation of transcription in other plant parts. Protein fusions demonstrate nuclear localization of INO, consistent with a proposed role as a transcription factor for this member of the YABBY protein family. Through its ability to inhibit expression of the endogenous *INO* gene and transgenes driven by P-INO, SUP is shown to be a negative regulator of INO transcription. Substitution of another YABBY protein coding region (*CRABS CLAW*) for *INO* overcomes this negative regulation, indicating that SUP suppresses *INO* transcription through attenuation of the *INO* positive autoregulatory loop.

Key words: YABBY, Reproductive development, Seed, Polarity, *Arabidopsis thaliana*

INTRODUCTION

The developmental processes that generate the plant body require specific induction and spatial confinement of regulatory gene expression. Clear examples of this are seen in the genes responsible for shoot meristem maintenance and floral organ specification (Bowman and Eshed, 2000; Ng and Yanofsky, 2000). Such regulation is also observed during lateral organ development where the specification of polarity along the abaxial (lower)-adaxial (upper) axis is essential for both organ growth and polar differentiation (Hudson, 2001). In *Arabidopsis*, the *PHABULOSA* (*PHB*) subfamily of homeodomain-leucine zipper proteins have been implicated in specification of adaxial identity (McConnell et al., 2001), and members of the YABBY and KANADI gene families are required for abaxial identity (Bowman, 2000; Eshed et al., 2001; Kerstetter et al., 2001). *PHB* and related genes are expressed on the adaxial side of developing leaves and appear to respond to signals from the shoot apical meristem. In contrast, expression of the YABBY and KANADI family members is confined to the abaxial side of lateral organs. Ectopic expression of these polarity determinants results in the ectopic differentiation of adaxial or abaxial cell types (McConnell et al., 2001; Sawa et al., 1999; Siegfried et al., 1999). Interestingly, initial expression of members of all three gene families appears to be present throughout the primordia anlagen, suggesting that they must subsequently be partitioned during organ development. To further understand the complex process of polarity determination and its role in lateral organ growth, we have focused on ovules, the precursors to seeds and the final structures produced during flower development.

Arabidopsis ovule morphogenesis superficially resembles shoot and flower development (Robinson-Beers et al., 1992; Schneitz et al., 1995). An axis (the ovule primordium) gives rise to two lateral organs (the integuments) from regions flanking the apex. The inner integument develops as a radially symmetrical structure that surrounds the terminal nucellus. In contrast, the outer integument is asymmetrical from its inception; it initiates only on the abaxial side of the ovule primordium (the side closest to the base of the gynoecium) and subsequently grows extensively from this side. The *Arabidopsis INNER NO OUTER* (*INO*) gene has been associated with both polarity determination and outer integument initiation in ovule development (Villanueva et al., 1999). *INO* encodes a putative transcription factor and is one of the six members of the YABBY gene family in *Arabidopsis*. *INO* mRNA initially accumulates only on the abaxial side of ovule primordia at the site of outer integument initiation, and subsequently in only the outer of the two cell layers of the developing outer integument (Balasubramanian and Schneitz, 2000; Villanueva et al., 1999). Strong *ino* mutants completely lack outer integuments and the absence of integument growth

was correlated with decrease in *INO* mRNA, implicating *INO* as a potential positive regulator of its own expression. Ovules of *superman* (*sup*) mutants have nearly equal growth of the outer integument on both the abaxial and adaxial sides of the ovule primordium (Gaiser et al., 1995). The ectopic growth of the outer integument was found to be associated with an apparent spread of *INO* mRNA to the adaxial side of the ovule in *sup* mutants and thus, *SUP* was hypothesized to be a negative regulator of *INO* expression (Villanueva et al., 1999).

We describe the identification of a region of the *INO* gene sufficient to reproduce the endogenous pattern of expression in transgenic plants. Reporter gene constructs utilizing this putative *INO* promoter enable more refined analysis of regulation of *INO* expression by allowing monitoring of transcription in mutants and transgenic plants. Use of the promoter for ectopic expression of *SUP* and another member of the YABBY family indicated that INO is involved in a positive autoregulatory circuit that is attenuated by SUP. This regulatory circuit is required for initiation and asymmetric growth of the *Arabidopsis* outer integument, supporting the strict requirement for spatial confinement of regulatory gene expression in lateral organ growth.

MATERIALS AND METHODS

Transgene vector construction

P-INO::INO:GUS::INO3′: A 6.5 kb S*al*I/*Eco*RI fragment containing the entire *INO* genomic coding sequence and extending into both adjacent open reading frames was isolated from bacterial artificial chromosome clone TAMU8E11 (Villanueva et al., 1999) and inserted into these same sites in pBJ61 (Gleave, 1992) as pSAO1. Site directed mutagenesis (Kunkel, 1985) was used to replace the INO stop codon with both *Nco*I and *Kpn*I restriction sites using the oligonucleotide INONCOKPN (GAAAATCTCCATTTGAGTCCATGGAATAG-GTACCAATTTGGGATATGA), producing pSAO7. The β-glucuronidase (GUS) coding sequence (Jefferson, 1987) from pHK7 (Harikrishna et al., 1996) was inserted into pSAO7 as a *Nco*I/*Kpn*I fragment creating pRJM66.

P-INO::GUS::INO3′: To isolate the INO5′-flanking region, the oligonucleotide INO5′BAMHISDM (CTCCTATCATTCATCG-GATCCACACACTCTCTATGAC) was used to introduce a *Bam*HI site upstream the putative INO start codon by site directed mutagenesis in pSAO1. The 2.3 kb *Sal*I/*Bam*HI fragment was inserted into these same sites in pBluescriptKS– (Stratagene, La Jolla, CA) creating pRJM25. The region 3′ of the INO stop codon was amplified from pSAO1 using the primers INOCHMK1 (GCTCTAGAGAGAA-GAGTCCTTGG) and M13reverse; the resulting 2.0 kb fragment was inserted into the *Xba*I/*Eco*RI digested pLITMUS28 (New England Biolabs, Beverly, MA) (pRJM06). The INO5′ fragment of pRJM25, the GUS coding sequence fragment from pHK7 and the INO3′ fragment of pRJM06 were assembled in pBJ61 forming pRJM65. The regions flanking the coding sequence are identical to those in the previously described pRJM33 (Villanueva et al., 1999).

P-INO::GUS::NOS3′: The *Sal*I/*Eco*RI fragment of pRJM65 comprising P-INO and GUS coding regions was inserted into pHK7, replacing the promoter and GUS coding sequence of that clone, creating pRJM77.

P-INO::INO:GFP::NOS3′: The INO cDNA (pRJM23) (Villanueva et al., 1999) was modified by PCR to introduce an *Xho*I site upstream of the start codon and to replace the stop codon with both *Pst*I and *Nco*I restriction sites using the primers INO5′XHOI (ATACTCGAGATGACAAAGCTCCCCAAC) and INO3′PSTINCOI (AATCCATGGCTGCAGCTCAAATGGAGATTTTCC). This 0.7 kb fragment was inserted into pLITMUS28, creating pRJM107. Using pRJM25, a *Hin*dIII and *Xho*I site were added at the 5′ and 3′ termini, respectively, of the INO5′ region by inserting double stranded oligonucleotides into existing restriction sites, creating pRJM192. The INO5′ fragment of pRJM192, the INO coding sequence of pRJM107 and the *Pst*I/*Kpn*I fragment of pRJM86 (containing the GFP1.1.5 coding sequence) (Schumacher et al., 1999) were assembled in pMON999 as pLMK20. pMON999 contained a modified cauliflower mosaic virus 35S promoter (35S) (Kay et al., 1987), which was removed in this cloning, and the polyadenlyation signal sequence of nopaline synthase (NOS3′) flanking a multiple cloning site.

35S::INO::NOS3′: The INO coding sequence of pRJM23 was transferred as a *Bam*HI/*Xba*I fragment into *Bgl*II/*Xba*I digested pMON999, creating pRJM64.

P-INO::SUP::INO3′: The INO coding sequence of pRJM33 was replaced with the 0.6 kb SUP cDNA fragment of pHS-SUPL1 (Sakai et al., 1995) using restriction enzymes *Bam*HI and *Xho*I (pRJM88).

P-INO::CRC::INO3′: *Bam*HI and *Xba*I restriction sites were added to the CRC coding sequence (Bowman and Smyth, 1999) 5′ and 3′ termini, respectively, by PCR using the primers 2567 (GGATC-CGCGGTTTTCAA) and CRCCHMJ2 (CTTCTAGACCAAAGGGA-CATAGCAAGTG) and the resulting product was cloned into pLITMUS28 as pRJM22. The 0.8 kb coding sequence fragment was used to replace the INO cDNA fragment of pRJM33 (pRJM45).

Plants and plant transformation

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

ino-1 and *ino-4* have been described previously (Villanueva et al., 1999). To create an *ino-1* (L*er*)/*INO* (Col) segregating population for transformation, an *ino-1* plant was crossed to a wild-type Col plant and heterozygous F_2 seed were collected. The genotype at the *INO* locus in F_3 progeny was determined using a Col/L*er* sequence polymorphism that is evaluated by PCR using the primers INOsslpfor (CCTTAACTGCTAAATGTAACCC) and INOsslprev (CAGCTGT-GTTTCTTTTTCCATC), which amplifies a fragment deriving from a location 4.8 kb 3′ of the *ino-1* lesion.

All transgenes were shuttled as *Not*I fragments into the plant transformation vector pMLBART (Gleave, 1992). Resulting plasmids were transferred into the *Agrobacterium tumefaciens* strain ASE (Fraley et al., 1985) by triparental mating (Figurski and Helinski, 1979). Plant transformation was performed as described previously (Clough and Bent, 1998) and transformants were selected for phosphoinothricine (BASTA) resistance.

Histochemical staining

Histochemical staining for β-glucuronidase activity (Jefferson, 1987) was performed in 25 mM KPO_4 (pH 7.0), 1.25 mM $K_3Fe(CN)_6$, 1.25 mM $K_4Fe(CN)_6$, 0.25 mM EDTA, 0.25% (v/v) Triton X-100, 20% (v/v) methanol containing either 12.5 μg/ml or 125 μg/ml (as indicated in the text) 5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexylamine salt (X-gluc) (Rose Scientific, Alberta, Canada). Prior to staining, plant material was fixed for 15 minutes in 90% acetone, followed by two washes in the assay solution (without X-gluc). Tissue was stained at 37°C for 15 hours and stored in 70% ethanol at 4°C.

Microscopy

Stained tissue was dissected, mounted in water and visualized using a Zeiss (Oberkochen, Germany) Axioplan microscope with differential interference contrast (DIC) optics. Confocal microscopy was performed on a Leica (Mannheim, Germany) TCS-SP scanning laser confocal microscope with differential interference contrast optics. Dissected tissue was mounted in water and GFP was excited using an argon laser (448 nm) and emission was monitored between 510-550 nm. Scanning electron microscopy was preformed as described previously (Broadhvest et al., 2000). Images were recorded

digitally and processed using Photoshop 6.0 software (Adobe Systems, San Jose, CA).

RESULTS

Reporter genes mimic endogenous INO expression pattern

Ovule development in *Arabidopsis* has been described previously (Robinson-Beers et al., 1992; Schneitz et al., 1995) and is briefly reviewed here (Fig. 1A-E). During stage 1 (stages from Schneitz et al., 1995), radially symmetrical ovule primordia initiate from placental tissue. An inner integument initiates from a ring of tissue encircling each primordium at stage 2-II, and at stage 2-III, the outer integument initiates from the abaxial side of the ovule primordium adjacent to the inner integument in a proximal (closer to the placenta) part of the ovule primordium. Both integuments grow towards the nucellus during stages 2-IV and 2-V and by stage 3-I the outer integument has covered the nucellus and inner integument. The asymmetric growth of the outer integument, favoring the abaxial side of the ovule primordium, results in the placement of the micropyle adjacent to the funiculus at stage 4-I.

Expression of *INO* is initially limited to a small region on the abaxial side of the ovule primordium and is subsequently confined within the developing outer integument as determined by in situ hybridization (Villanueva et al., 1999). To extend these observations and to determine the subcellular localization of INO, we assembled a translational fusion of the *INO* coding sequence with the green fluorescent protein of *Aequorea victoria* (GFP) (Haseloff et al., 1997) under control of a putative INO promoter (P-INO::INO:GFP::NOS3′). Using confocal laser scanning microscopy, GFP was detectable in five of ten primary Ino^{+} transformants (either with at least one endogenous *INO* allele or homozygous *ino-1* complemented by the translational fusion) (Fig. 1F-J). INO:GFP was first visible

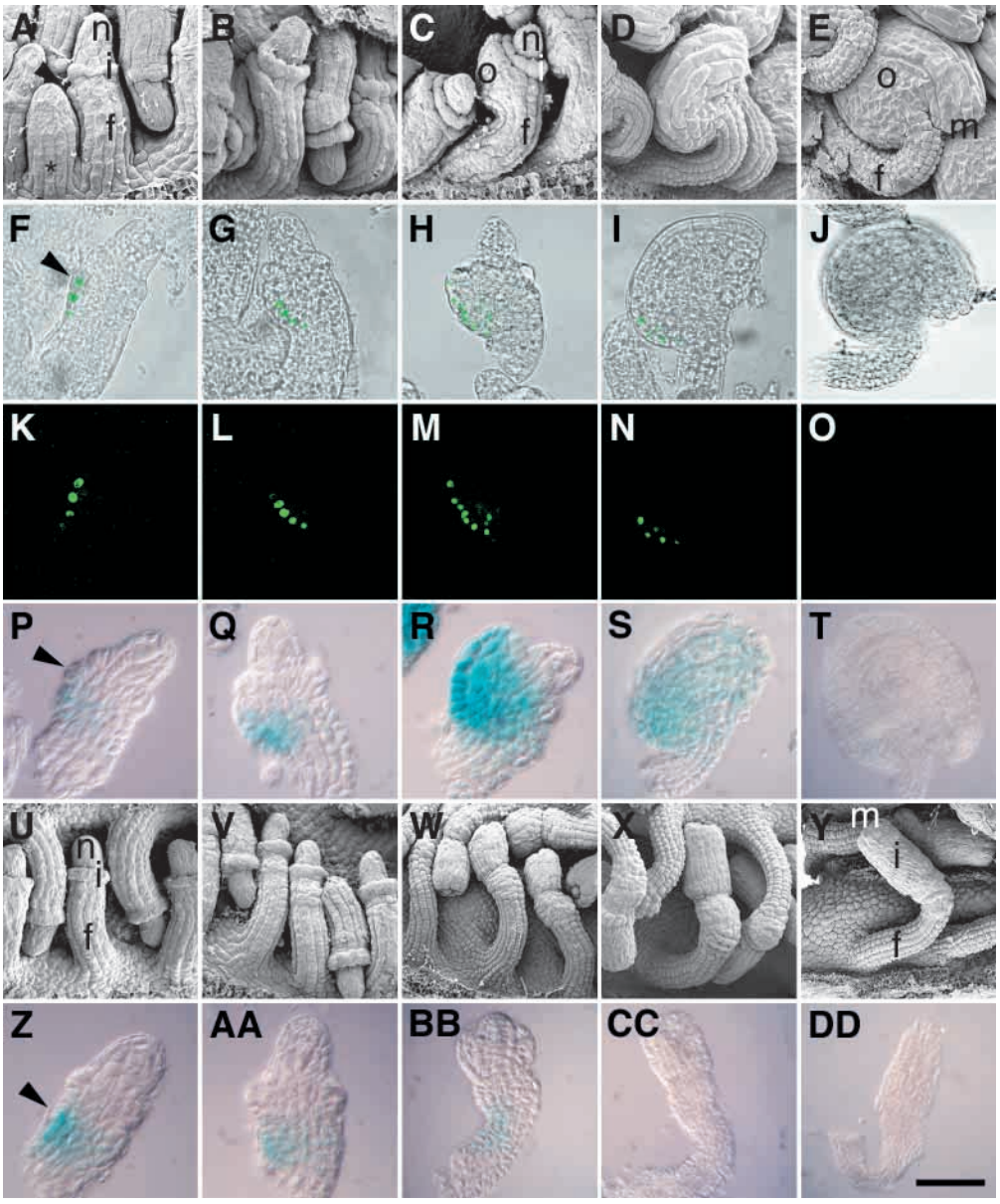

Fig. 1. Expression of P-INO reporter genes in wild type and *ino-1* plants. Stages: (A,F,K,P,U,Z) 2-III; (B,G,L,Q,V,AA) 2-IV; (C,H,M,R,W,BB) 2-V; (D,I,N,S,X,CC) 3-I; (E,J,O,T,Y,DD) 4-I. (A-E) Scanning electron micrographs of wild-type ovules. In A a late developing ovule at stage 1 is indicated with an asterisk. (F-J) Composite figures of DIC and confocal INO:GFP fluorescence images and (K-O) respective INO:GFP fluorescence alone in complemented *ino-1* ovules. P-INO::INO:GFP::NOS3′ fluorescence is detectable only on the abaxial side of the ovule primordium and the abaxial layer of the outer integument. (M,N) Apparent internal localization of INO:GFP is from glancing sections through adjacent surface layers. (P-T) DIC images of wild-type ovules of P-INO::GUS::INO3′ transgenic plants stained for GUS activity using 12.5 μg/ml X-gluc. (U-Y) Scanning electron micrographs of *ino-1* ovules. (Z-DD) *ino-1* ovules stained for P-INO::GUS::INO3′ activity using 125 μg/ml X-gluc and visualized using DIC light microscopy. In all panels, the abaxial side (toward the base of the gynoecium) of the ovule is to the left, and adaxial is towards the right. n, nucellus; i, inner integument; f, funiculus; o, outer integument; m, micropyle; arrowhead, outer integument primordium. Scale bar: 25 μm in A-C,F-H,K-M,P-R,U-W,Z-BB; 30 μm in D,I,N,S,X,CC; 50 μm in E,J,O,T,Y,DD.

at stage 2-II in the nuclei of cells in a region of the chalaza (the region giving rise to the integuments) three cell layers wide on the abaxial side and progressively narrowing to a single cell in width approximately two-thirds of the way around the primordium. These cells demarcated not only the basal region of the future site of outer integument initiation but also appeared to include some subtending cells on the abaxial side of the ovule primordium. These subtending cells were either incorporated into the outer integument or ceased *INO* expression because by stage 2-IV, the proximal cells adjacent the outer integument did not contain detectable GFP. From stage 2-IV until stage 3-I, INO:GFP was localized exclusively to the outer cell layer of the outer integument but was never visible in any cells on the extreme adaxial side of the ovule primordium. At stage 3-I, INO:GFP was further confined to the chalazal end of the integument before becoming undetectable. This pattern duplicates that observed for accumulation of *INO* mRNA, indicating that the 2.1 kb of *INO* 5′-flanking sequence contains sufficient regulatory information to reproduce the endogenous spatial and temporal expression pattern of *INO*. We designate this fragment as the INO promoter, P-INO.

To confirm the P-INO::INO:GFP::NOS3′ expression data and create a more easily assayed reporter construct, both translational and transcriptional fusions of the coding sequence of *E. coli* β-glucuronidase (GUS) to P-INO were assembled. The translational fusion, P-INO::INOgen:GUS::INO3′, included regions both upstream and downstream of the genomic INO coding sequence in addition to all endogenous introns. Transcriptional fusions used P-INO to drive production of GUS in conjunction with either the putative endogenous INO polyadenylation signal sequence, (P-INO::GUS::INO3′) or the NOS3′ sequence (P-INO::GUS::NOS3′). GUS activity for each construct was examined in Ino^+ plants (either with at least one endogenous *INO* allele or homozygous *ino-1* complemented by the translational fusion) and was found to be indistinguishable among the three transgenes, closely mimicking endogenous *INO* expression and the INO:GFP transgene (Fig. 1P-T and data not shown). GUS activity was first observed at stage 2-III, in the few cells that form and subtend the outer integument. During the following developmental stages, GUS staining was restricted to the outer integument, but appeared to extend to the adaxial side of the ovule primordium by stage 2-V. Activity dropped to an undetectable level by stage 4-I. Expansion of GUS activity into the funiculus, inner integument or nucellus was not observed. Thus, staining for GUS activity appeared to initiate slightly later, extend further around the ovule primordium, and persist longer than signals detected in either in situ analysis of *INO* mRNA or confocal analysis of the P-INO::INO:GFP::NOS3′ transgene. Because these differences can be accounted for by a combination of GUS protein stability and diffusion of the primary enzymatic product of GUS (Jefferson, 1987), the P-INO::GUS::INO3′ transgene provided an effective means of monitoring expression from P-INO in the presence or absence of functional INO.

In summation, expression from P-INO was found to be confined to the abaxial side of the ovule primordium, and to the cells giving rise to and subsequently constituting the outer layer of the outer integument. The nuclear localization of the INO:GFP protein and the ability of the P-INO::INO:GFP::NOS3′ transgene to complement the *ino-1* mutation provides further evidence that the nucleus is the normal site of action of INO, consistent with its previously hypothesized role as a transcription factor (Villanueva et al., 1999).

Reduced P-INO::GUS expression in *ino-1*

Effects of known *ino* alleles are limited to ovule development (Villanueva et al., 1999) (Fig. 1U-Y). In *ino-1* mutant plants, ovule development is similar to wild-type development until stage 2-III, at which time the outer integument fails to initiate. The inner integument is unaffected and envelops the nucellus by stage 3-I. In the absence of an outer integument, the ovules remain largely erect and the micropyle is not positioned near the funiculus.

Based on a lack of detectable INO transcript in in situ hybridizations of the strong *ino-1* mutant, we previously hypothesized that either INO was a positive regulator of its own expression or that the *ino-1* mutation led to a reduced *INO* transcript stability (Villanueva et al., 1999). To distinguish between these two hypotheses, P-INO::GUS::INO3′ expression was analyzed in a homozygous *ino-1* background. Since this transgene does not contain the *ino-1* mutant coding sequence, any alterations in expression should be due to changes in expression level through the promoter. With the concentration of the GUS substrate used for analysis of wild-type plants, GUS activity from the P-INO::GUS::INO3′ transgene was undetectable at any stage in *ino-1* mutants. However, when the substrate concentration was increased ten-fold, GUS activity was detectable (Fig. 1Z-1DD). Activity was first observed at stage 2-III in only the abaxial side of the ovule primordium, the same location where GUS initially accumulated in wild-type ovules. GUS activity persisted in this location until stage 2-V, but expansion of GUS activity to the adaxial side of the ovule primordium was not observed. Thus, although P-INO::GUS::INO3′ expression is initiated at the correct time and location in *ino-1* mutants, expression was reduced and less persistent relative to that in wild type. This shows that a positive influence of INO on P-INO is necessary to achieve the endogenous expression profile.

INO is not sufficient to activate ectopic expression of a P-INO::GUS transgene

As shown above, active INO can positively affect expression from the P-INO::GUS::INO3′ transgene within the ovule. To determine if INO can promote ectopic expression from the INO promoter, plants containing a transgene for the ectopic expression of *INO* from the cauliflower mosaic virus 35S promoter (35S::INO::NOS3′) were produced. Three classes of phenotypes were apparent in these plants (Fig. 2). In one class, the plants appeared unaffected, except for the ovules, which resembled those of plants with either the strong *ino-1* or weak *ino-4* alleles (data not shown). In a second class, only the leaves of the plants were affected. Both rosette and cauline leaves were curled and often also narrow or misshapen. The final class also had the leaf morphology defects but this was coupled with alterations in floral organ number and identity. In plants of this class, flowers could have supernumary organs in the outer three whorls, with the third whorl most severely affected, and a reduction or absence of fourth whorl tissue. In addition, the inflorescence had reduced internode length between flowers, resulting in a compact inflorescence structure. In plants with

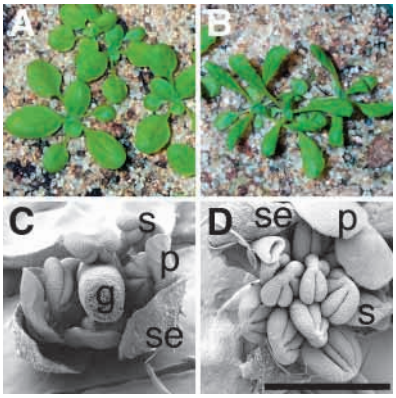

Fig. 2. Phenotypic effects of ectopic INO expression. (A) Four-week old wild-type plants; leaves are relatively flat. (B) 35S::INO::NOS3′ transgene in wild type background; leaves curl towards the abaxial (lower) side. (C) Wild-type flowers comprise four concentric whorls of floral organs: sepals, petals, stamens and a central gynoecium. (D) Flowers of one class of 35S::INO::NOS3′ transgenic plants produced supernumary stamens and a gynoecium was absent. se, sepal; p, petal; s, stamen; g, gynoecium. Scale bar: 30 mm in A,B; 1 mm in C,D.

similar phenotypes resulting from a 35S::INO:GUS::INO3′ transgene, the morphological changes were always associated with detectable GUS activity, showing that they resulted from ectopic production of INO (data not shown). Plants from the final class of 35S::INO::NOS3′ transgenics were crossed to a P-INO::GUS::INO3′ transgenic line. In examination of several progeny with the ectopic expression phenotypes and the P-INO::GUS::INO3′ transgene (confirmed by PCR), GUS activity was not detectable outside of ovules at any stage of leaf or flower development (data not shown). These results indicate that INO is not sufficient to initiate expression from P-INO in either flower (excluding ovules) or leaf tissue.

Expression from P-INO expands in *sup-5*

Mutations in *SUP* affect both flower and ovule development (Bowman et al., 1992; Gaiser et al., 1995; Schultz et al., 1991) (Fig. 3A-E). *sup-5* flowers have supernumerary stamens and frequently produce fourth whorl floral organs with both stamen and carpel characteristics. Early ovule development in *sup-5* is similar to that in wild type; at stage 2-II the symmetric ring of the inner integument primordium initiates, and the outer integument initiates from the abaxial side of the ovule primordium at stage 2-III. The first noticeable deviation from wild type occurs at approximately stage 2-V when *sup-5* ovules initiate outer integument growth from the adaxial side of the ovule primordium. In the resulting stage 4-I ovules, most of the outer integument is radially symmetrical and the micropyle is not adjacent to the funiculus.

Using in situ hybridization, late in ovule development *INO* mRNA appeared to be present in outer integument cells on both the abaxial and adaxial sides of the *sup-5* ovule primordium, and in the funiculus (Villanueva et al., 1999). This implied that SUP may inhibit growth of the outer integument by inhibiting the level or pattern of *INO* expression (Villanueva et al., 1999). To further explore this hypothesis, expression of the P-INO::INO:GFP::NOS3′ transgene was analyzed in the *sup-5* background (Fig. 3F-O). At stage 2-II, expression of INO:GFP in *sup-5* was indistinguishable from that in wild type. By stage 2-V, although restricted to the outer cell layer of the outer integument, INO:GFP had expanded across the chalaza and

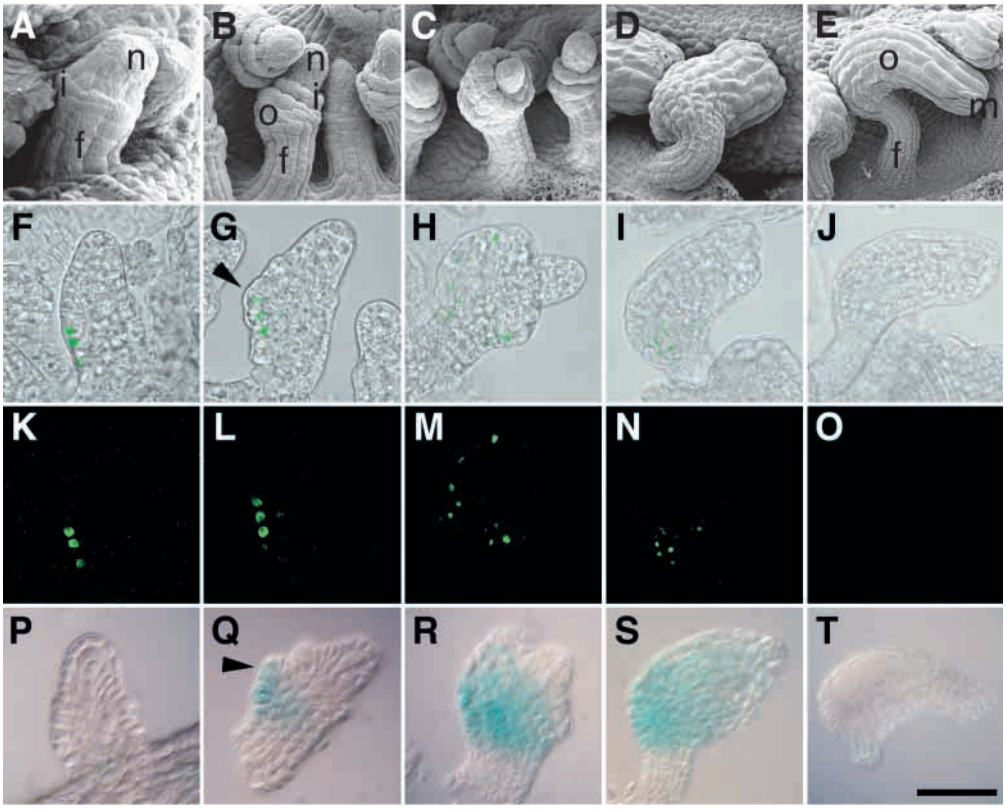

Fig. 3. Expression of P-INO reporter genes in *sup-5*. Stages: (A,F,K,P) 2-II; (B,G,L,Q) 2-IV; (C,H,M,R) 2-V; (D,I,N,S) 3-I; (E,J,O,T) 4-I. (A-E) Scanning electron micrographs of *sup-5* ovules. (F-J) Composite figures of DIC and confocal INO:GFP fluorescence images and (K-O) respective INO:GFP fluorescence alone in *sup-5* ovules. Although P-INO::INO:GFP::NOS3′ fluorescence is limited to a single cell layer, it expands to the adaxial side of the ovule primordium. (M,N) Apparent internal localization of INO:GFP is from glancing sections through adjacent surface layers. (P-T) DIC images of *sup-5* ovules stained for P-INO::GUS::INO3′ activity using 12.5 μg/ml X-gluc. In all panels, the abaxial side of the ovule is to the left. n, nucellus; i, inner integument; f, funiculus; o, outer integument; m, micropyle; arrowhead, outer integument primordium. Scale bar: 25 μm in A-C,F-H,K-M,P-R; 30 μm in D,I,N,S; 50 μm in E,J,O,T.

was detected in the outer integument on both the abaxial and adaxial sides of the ovule primordium. Expansion of INO:GFP fluorescence into the funiculus was not observed. Expansion of GUS activity to the adaxial side of the ovule primordium was also observed with the P-INO::GUS::INO3′ transgene in *sup-5* (Fig. 3P-T). These results indicate that SUP is not required for the correct initiation or upregulation of INO expression, but that it is essential to prevent the expansion of *INO* expression to the adaxial side of the ovule primordium. SUP does not appear to participate in confining INO:GFP to the abaxial layer of the integument because this pattern was maintained in *sup-5*.

To determine if active INO is necessary for the adaxial expression from P-INO in *sup-5*, activity of the P-INO::GUS:INO3′ transgene was analyzed in the *ino-1 sup-5* double mutant. The *ino-1* mutation is epistatic to *sup-5* in ovule morphogenesis and ovules of these plants resemble *ino-1* mutant ovules (the floral phenotype of *sup-5* is unaffected by *ino-1*) (Gaiser et al., 1995). P-INO::GUS::INO3′ activity in the *ino-1 sup-5* double mutant duplicated that seen in the *ino-1* single mutant (data not shown). Detection required the elevated concentration of X-gluc, and activity was confined to the abaxial side of the ovule primordium at all stages where it was detectable. This demonstrates that active INO is required for the expansion of *INO* expression across the ovule primordium in *sup-5* and that the initial confinement of expression from P-INO to the abaxial side of the ovule primordium is not dependent on SUP activity.

P-INO::SUP can phenocopy effects of *ino* mutations

SUP appears to function as a negative regulator of integument growth and *INO* expression on the adaxial side of the ovule primordium. To test the hypothesis that SUP might be sufficient to inhibit these processes, a transcriptional fusion of the *SUP* coding sequence to the *INO* promoter (P-INO::SUP::INO3′) was assembled and introduced into wild-type plants (Fig. 4A-E). As in wild type, in 15 primary transgenic plants, the outer integument initiated at stage 2-III and formed a small ridge of tissue on the abaxial side of the ovule primordium. In contrast to wild type, in all but one of the transformants, outer integument growth ceased by stage 2-IV, and at stage 4-I the small ridge of tissue did not cover any portion of the inner integument. These ovules superficially resembled stage 4-I ovules of *ino-1* plants. In one transformant, the outer integument grew to partially cover the inner integument by stage 4-I and therefore resembled the weaker *ino-4* allele (Villanueva et al., 1999). Growth of this rudimentary outer integument in the P-INO::SUP::INO3′ plants was dependent on the production of active INO; homozygous *ino-1* plants (nine total) that contained the transgene did not initiate outer integument growth. Accumulation of GUS activity from the P-INO::GUS::INO3′ transgene in Ino$^+$ plants containing the P-INO::SUP::INO3′ transgene was essentially identical to that observed in *ino-1* mutants, being first apparent at stage 2-III at the site of outer integument initiation and persisting within the arrested outer integument only until stage 2-V (Fig. 4F-J). These results demonstrate that production of SUP on the abaxial side of the ovule primordium is sufficient to inhibit INO-dependent outer integument growth and *INO* expression but does not obstruct integument initiation. Therefore, SUP can function directly in cells where it is transcribed and is not dependent upon factors specific to the adaxial side of ovule primordia.

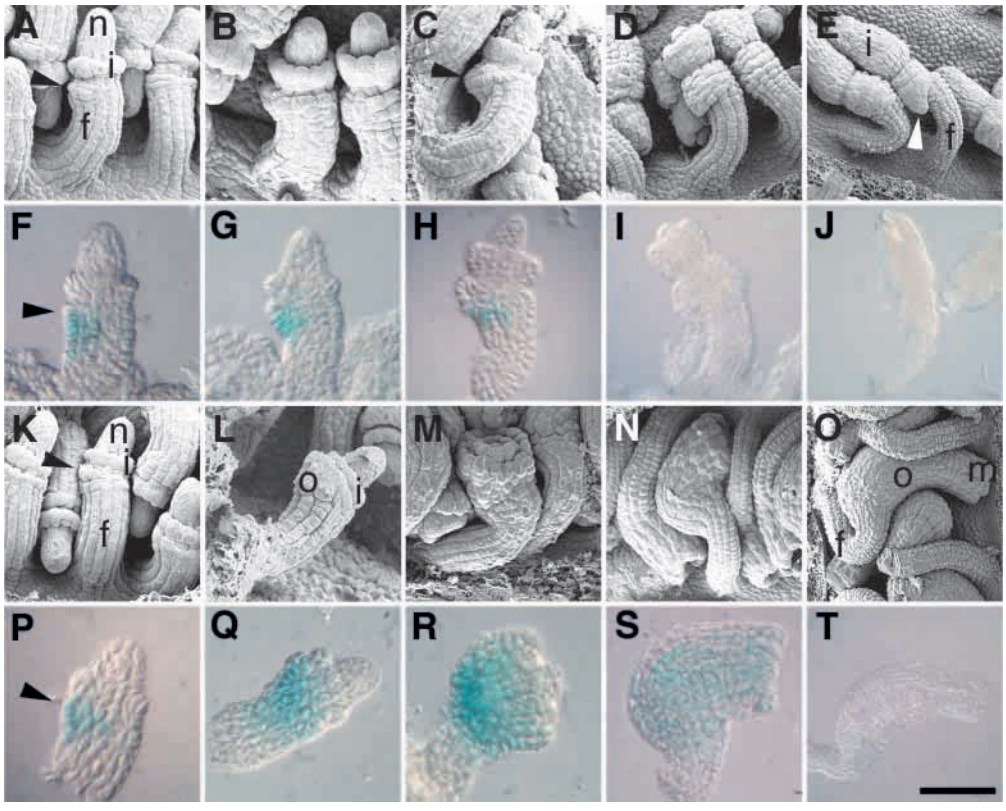

Fig. 4. Effects of P-INO::SUP::INO3′ and P-INO::CRC::INO3′ transgenes on ovule morphology and reporter gene expression. Stages: (A,F,K,P) 2-III; (B,G) early 2-IV; (C,H,L,Q) 2-IV; (D,I,M,R) 2-V; (N,S) 3-I; (E,J,O,T) 4-I. (A-E) Scanning electron micrographs of ovules from wild-type plants containing the P-INO::SUP::INO3′ transgene. (F-J) DIC images of P-INO::SUP::INO3′ ovules stained for P-INO::GUS::INO3′ activity using 125 μg/ml X-gluc. (K-O) Scanning electron micrographs of ovules from *ino-1* plants containing the P-INO::CRC::INO3′ transgene. (P-T) DIC images of P-INO::CRC::INO3′ ovules from *ino-1* plants stained for P-INO::GUS::INO3′ activity using 12.5 μg/ml X-gluc. In all panels, the abaxial side of the ovules is to the left. n, nucellus; i, inner integument; f, funiculus; o, outer integument; m, micropyle; arrowhead, outer integument primordium. Scale bar: 25 μm in A-D,F-I,K-M,P-R; 30 μm in N,S; 50 μm in E,J,O,T.

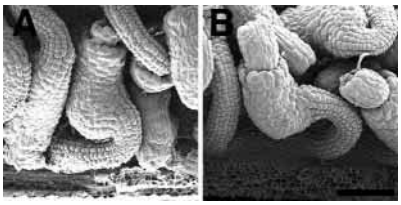

Fig. 5. Stage 4-I ovules from *ino-1* plants containing both the P-INO::SUP::INO3′ and P-INO::CRC::INO3′ transgenes. Scanning electron micrographs of two progeny from a single cross representing the two classes of ovules; (A) *sup*-like and (B) *ino-4*-like. Scale bar: 50 μm.

P-INO::CRC can overcome inhibitory effects of SUP

CRABS CLAW (*CRC*), another member of the *Arabidopsis* YABBY gene family, serves as a mediator of polar and homeotic development of the carpel, is essential for nectary formation, and its expression is limited to those structures (Bowman and Smyth, 1999). To determine if CRC was functionally equivalent to INO and therefore able to complement the *ino-1* mutation, a transcriptional fusion of P-INO to the CRC coding sequence was produced (P-INO::CRC::INO3′). Of 25 independent transformants containing the P-INO::CRC::INO3′ transgene examined in an *ino-1* background, five showed a phenotype that was similar to *sup-5* mutant ovules (Fig. 4K-O) and 15 displayed a reduced but identifiable *sup*-like phenotype, as evaluated by the amount of outer integument growth from the adaxial side of the ovule primordium. Ovules of four transformants resembled *ino-4* ovules with the outer integument only partially covering the inner integument at anthesis, and ovules of the final plant were like those of *ino-1* plants. As in *sup-5* mutant ovules, asymmetric initiation of the outer integument was followed by symmetrical growth starting at stage 2-V in the most strongly affected plants. Activity of the P-INO::GUS::INO3′ transgene was similar to that seen in the *sup-5* mutant when examined in *ino-1* plants containing the P-INO::CRC::INO3′ transgene (Fig. 4P-T). Elevated GUS substrate was not required and GUS activity was first detected at the site of outer integument initiation at stage 2-III on the abaxial side of the ovule primordium. GUS activity expanded to the adaxial side of the ovule primordia by stage 2-V and was present in the region of outer integument that originated from the adaxial side of the ovule primordium. Thus, the P-INO::CRC::INO3′ transgene could produce a phenocopy of *sup-5* in both ovule morphogenesis and expression from the *INO* promoter in spite of the presence of active SUP.

To determine if expression of CRC could overcome inhibition of integument growth by ectopic *SUP* expression, *ino-1* plants containing both the P-INO::CRC::INO3′ and P-INO::SUP::INO3′ transgenes were isolated. In ovules of five of the 18 progeny examined, growth of the outer integument resembled that of plants containing only the P-INO::CRC::INO3′ transgene; the outer integument grew from both the abaxial and adaxial sides of the ovule primordium and phenocopied *sup-5* (Fig. 5A). Ovules from the remaining progeny resembled those of *ino-4*, with the outer integument only partially covering the inner integument, but unlike *ino-4*, growth from the adaxial side of the primordium did occur (Fig. 5B). The increased integument growth relative to plants lacking the CRC transgene was not due to a genetic reduction of the P-INO::SUP::INO3′ transgene because the parental plants were also crossed to wild-type plants and the strong P-INO::SUP::INO3′ phenotype was apparent in all progeny. Thus, the inhibitory effects of P-INO::SUP::INO3′ on integument growth can be overcome by the P-INO::CRC::INO3′ transgene.

These results show that CRC can substitute for INO in the promotion of outer integument growth and can overcome the inhibitory effects of SUP from either endogenous or P-INO-driven expression. Because the only difference between the P-INO::INO::INO3′ and P-INO::CRC::INO3′ transgenes was the coding region, these results indicate that the coding region must play a role in negative regulation by SUP, which could be manifested through either the protein produced by the transgene, or through possible regulatory binding sites within the coding sequence.

DISCUSSION

INO is required for upregulation and maintenance of asymmetric *INO* expression

Previous work (Balasubramanian and Schneitz, 2000; Villanueva et al., 1999) and our reporter gene analysis shows that *INO* expression is spatially confined along two different axes. It is restricted to the abaxial side of the axis of the developing ovule, and to the abaxial layer of the outer integument. The earlier work also indicated that the level of endogenous *INO* transcript was reduced in both the strong *ino-1* and weak *ino-4* mutants (Villanueva et al., 1999). Our observations using the P-INO::GUS::INO3′ reporter gene confirm these results and show that active INO is required for maintenance and up-regulation of expression driven by P-INO, but is not essential for the initial transcription from P-INO. Because ectopic expression of INO did not induce P-INO::GUS::INO3′ expression in either leaves or flowers, we conclude that INO is not sufficient for de novo activation of transcription from P-INO, but requires an additional ovule-specific factor for initial activation. Our results do not allow us to determine if INO acts directly on P-INO, or if it acts through other factors.

SUP suppresses *INO* expression

SUP was previously hypothesized to function as a negative regulator of INO expression owing to an observed expansion of *INO* mRNA accumulation to the adaxial side of *sup* ovules (Villanueva et al., 1999). Our demonstration that the P-INO::SUP::INO3′ transgene was sufficient to reduce integument growth and expression from P-INO confirms this hypothesis. The observation that SUP was effective in reducing expression from P-INO reporter genes indicates that the negative regulation is manifest through the INO promoter region, and therefore must involve regulation of transcription. The recent demonstration that SUP includes a transcription repression domain (Hiratsu et al., 2002) is consistent with this proposed role for SUP. However, endogenous *SUP* mRNA accumulation has only been reported in the funiculus in an area adjacent to, but not overlapping with, the chalazal region where SUP appears to suppress *INO* expression (Sakai et al., 1995). Thus, SUP appears to exhibit non-cell autonomous activity in

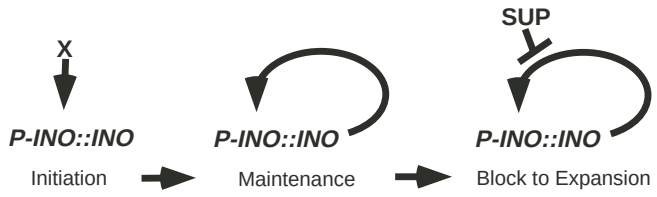

Fig. 6. Model of the transcriptional regulation of INO. Unknown factor 'X' initiates transcription from P-INO prior to outer integument initiation. INO maintains and enhances expression from P-INO. SUP blocks the INO autoregulatory loop of incipient expression on the adaxial side of the ovule preventing detectable expression and integument growth in this zone.

its endogenous function within the ovule but our ectopic expression results show that it can also inhibit *INO* expression and integument growth in the cells in which it is transcribed. The epistasis of *ino* to *sup* in ovule development (Gaiser et al., 1995), and the complete correlation between the effects of SUP on *INO* expression and integument growth imply that all effects of *sup* mutations on ovule development are manifest through alterations in *INO* expression.

SUP inhibits integument growth by affecting INO autoregulation

The transcriptional regulation of INO is influenced by apparently antagonistic actions of INO and SUP. However, this relationship can be altered by changes to the INO coding sequence as evidenced by the ability of CRC to overcome the endogenous function of SUP. A model that explains these results is shown in Fig. 6. INO activates transcription from P-INO (possibly indirectly), and SUP inhibits this activation. Thus, in wild-type ovules, inhibition of INO autoregulation by SUP would block perpetuation of hypothesized incipient *INO* expression (expression that is undetectable by INO:GFP or in situ analysis) on the adaxial side of the ovule, maintaining the established asymmetric pattern of *INO* expression and outer integument growth. However, since CRC is less sensitive to the effects of SUP, the hypothesized incipient expression allows for activation of the autoregulatory pathway and subsequent growth of the outer integument. In plants harboring the P-INO::SUP::INO3′ transgene, some INO is produced on the abaxial side of the ovule, due to simultaneous induction of expression, initiating integument growth. However, subsequent growth would be inhibited by the action of SUP in blocking perpetuation of *INO* expression.

The phenotype produced by the P-INO::SUP::INO3′ transgene is nearly identical to that observed for the weak *ino-4* allele. The *ino-4* mutation results in the addition of five amino acids to the conserved YABBY region, the putative DNA binding domain. Thus, nearly identical phenotypes result from low-level transient expression of wild-type *INO* and initially normal expression of a compromised protein.

While our model can explain the effects of regulatory interactions between *INO* and *SUP*, the molecular mechanisms underlying these interactions remain unclear. The conceptually simplest mechanism would be for INO to directly interact with and activate transcription from P-INO. SUP would inhibit autoregulation by interfering with the binding of INO to P-INO or activation of transcription by bound INO. CRC would bind or activate more effectively than INO in the presence of SUP. Alternatively, the actions of both INO and SUP on *INO* expression may be less direct. SUP has been proposed to be a negative regulator of growth (Sakai et al., 2000; Sakai et al., 1995). INO may be a promoter of growth and require a growth-competent state for maintenance of its expression. Thus promotion of growth would be a part of the INO autoregulatory loop, and suppression of growth, or growth competency, by SUP would be the mechanism by which SUP inhibits *INO* expression. CRC would be a stronger promoter of growth than INO and would thus be able to more effectively compete with SUP. Both of these mechanisms still have SUP as a formal negative regulator of INO transcription and are consistent with the proposed model. Because the direct regulation mechanism predicts interactions of INO and CRC with P-INO, and interactions of SUP with either P-INO or INO, it can be directly tested by performing experiments to detect such interactions. The tight linkage between *INO* expression and growth makes the second mechanism more difficult to test.

We note that our model addresses only the regulation of spatial distribution of *INO* expression across the width of the chalazal region during integument initiation and growth. The confinement of *INO* expression to a single layer of the outer integument is maintained in *sup* mutants, indicating that this confinement is under control of other factors. It is possible that *INO* autoregulation is also important in abaxial expression within the integument, and that other factors, with activities analogous to SUP, interfere with this autoregulation to maintain the pattern of expression.

INO and polarity determination

We previously proposed that INO was one determinant of abaxial chalazal identity within the ovule primordium, with extensive outer integument growth being a characteristic feature of that region. The precise correlation between asymmetric expression of *INO* in the ovule primordium and asymmetric growth of the integument in the current study is consistent with this hypothesis. We also now note asymmetric *INO* expression within the outer integument. From its earliest appearance, INO:GFP is present only in the abaxial cells of the outer integument anlagen and remains confined to the abaxial layer of the outer integument throughout development. This supports the hypothesis that INO is also functioning in determination of abaxial identity of the outer integument and could provide an explanation of the tight linkage between *INO* activity and outgrowth of this structure. Based on observations of mutations affecting polarity determination in leaves of *Antirrhinum*, Waites and Hudson (Waites and Hudson, 1995; Waites et al., 1998) proposed a model for lateral organ growth in which the juxtaposition of abaxial and adaxial identity is essential for both laminar and proximal-distal outgrowth. If INO is required to specify abaxial identity of the integument, the loss of an adaxial-abaxial boundary due to the absence of INO activity would result in the failure of the outer integument to extend; growth of the outer integument could then be likened to the laminar or proximal-distal extension of other aerial lateral organs. This model predicts that other mutations affecting polarity of the outer integument would lead to a reduction in its growth. Indeed, *Arabidopsis* plants heterozygous for the gain-of-function *phb-1d* mutation or homozygous for the *kanadi1 kanadi2* double mutation have

reduced outer integument growth (McConnell and Barton, 1998; Eshed et al., 2001). Thus, similar to other lateral organs, outer integument growth must have a strict requirement for correct specification and juxtaposition of polarity.

Abaxial expression of at least one YABBY gene is a common feature shared by leaves and lateral floral organs of *Arabidopsis* (Siegfried et al., 1999), consistent with the possible common evolutionary origin of these structures (Gifford and Foster, 1989). The abaxial expression of *INO* in the outer integument may also indicate an evolutionary link between the outer integument and leaves or leaf-derived structures.

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