Independence and Interaction of Regions of the INNER NO OUTER Protein in Growth Control during Ovule Development[1][W][OA]

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The outer integument of the Arabidopsis (*Arabidopsis thaliana*) ovule develops asymmetrically, with growth and cell division occurring primarily along the region of the ovule facing the base of the gynoecium (gynobasal). This process is altered in the mutants *inner no outer* (*ino*) and *superman* (*sup*), which lead to absent or symmetrical growth of the outer integument, respectively. *INO* encodes a member of the YABBY family of putative transcription factors, and its expression is restricted to the gynobasal side of developing ovules via negative regulation by the transcription factor SUP. Other YABBY proteins (e.g. CRABS CLAW [CRC] and YABBY3 [YAB3]) can substitute for INO in promotion of integument growth, but do not respond to SUP regulation. In contrast, YAB5 fails to promote integument growth. To separately investigate the growth-promotive effects of INO and its inhibition by SUP, domain swaps between INO and YAB3, YAB5, or CRC were assembled. The ability of chimeric YABBY proteins to respond to SUP restriction showed a quantitative response proportional to the amount of INO protein and was more dependent on C-terminal regions of INO. A different response was seen when examining growth promotion where the number and identity of regions of INO in chimeric YABBY proteins were not the primary influence on promotion of outer integument growth. Instead, promotion of growth required a coordination of features along the entire length of the INO protein, suggesting that intramolecular interactions between regions of INO may coordinately facilitate the intermolecular interactions necessary to promote formation of the outer integument.

The YABBY family of genes participates in the specification of abaxial identity in plant lateral organs (Siegfried et al., 1999). *INNER NO OUTER* (*INO*) is an Arabidopsis (*Arabidopsis thaliana*) YABBY family member that is specifically expressed in the ovule outer integument and is essential for ovule development (Baker et al., 1997; Villanueva et al., 1999). *INO* likely modulates the transcription of currently uncharacterized genes involved in the growth of the outer integument in Arabidopsis. While the molecular basis of INO function remains largely unknown, the ability of INO to interact with the putative transcription factor NOZZLE/SPOROCYTELESS (Sieber et al., 2004) further supports the hypothesis that INO modulates expression of genes necessary for outer integument development. Structural domains of YABBY members have been hypothesized based on sequence alignments that show similarities to known protein domain motifs (Siegfried et al., 1999; Villanueva et al., 1999; Bowman, 2000). A region toward the N terminus is similar to Cys_2-Cys_2 zinc (Zn)-finger domains and has been shown to interact with Zn ions in vitro for the YABBY protein FILAMENTOUS FLOWER (Kanaya et al., 2001). A region (the ''YABBY'' domain) nearer to the C terminus is predicted to contain two α-helical regions that are thought to participate in DNA binding, in large part due to sequence similarity with the DNA-binding motif of the High Mobility Group transcription factors (Sawa et al., 1999). An understanding of the functions of these regions and a more precise determination of the boundaries of each putative domain would facilitate our understanding of how INO mediates outer integument growth in Arabidopsis.

During Arabidopsis ovule development, outer integument growth is restricted primarily to the side of the ovule that faces the basal region of the gynoecium (gynobasal; Fig. 1). This asymmetry is mediated via SUPERMAN (SUP), a transcription factor that has been shown to restrict *INO* expression to the gynobasal side of the developing ovule (Meister et al., 2002). While *ino* mutants failed to initiate or support outer integument growth, it was found that *sup* mutants resulted in expansion of *INO* expression to all sides of the developing outer integument. This resulted in outer integument growth on both the gynobasal and gynoapical (toward the stigma) sides of the ovule (Fig. 1). This ectopic growth correlated with gynoapical expansion of expression of *INO* and of *GFP* and β-glucuronidase (*GUS*) coding regions under control

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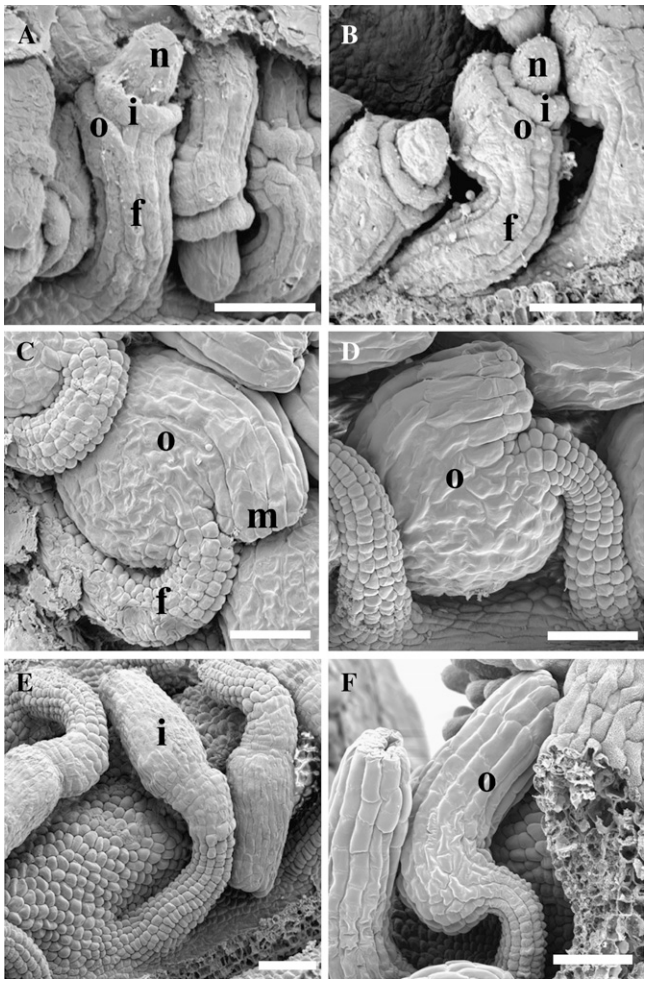

Figure 1. Scanning electron micrographs of wild-type and mutant ovules. During wild-type ovule development, primordia emergence is followed by the appearance of distinct structures, including the funiculus, outer and inner integuments, and the nucellus (A). Asymmetric growth is observed as the ovule develops (B) until the micropyle lies adjacent to the funiculus in mature ovules (C and D). In *ino-1* ovules (E), the outer integument fails to initiate and as a result, the symmetrical growth of the inner integument and nucellus are apparent. In *sup-5* ovules (F), the outer integument exhibits growth on both sides, leading toward a concentric ring of symmetrical growth. c, Chalaza; i, inner integument; m, micropyle; n, nucellus; o, outer integument. For all panels, the gynobasal side is at left. Scale bars = 25 μm.

of the *INO* promoter (PRO_{INO}), indicating that SUP repression of *INO* expression was mediated at the transcriptional level (Meister et al., 2002). To further test the nature of INO and SUP antagonism, Meister et al. (2002) placed the coding sequence of *CRABS CLAW* (*CRC*), a related YABBY member, under control of PRO_{INO} and introduced this into *ino-1* plants. Plants expressing PRO_{INO}:*CRC* produced ovules that phenocopied the *sup* mutant, and reporter analysis indicated expansion of PRO_{INO}-driven expression similar to that observed for *sup* mutant ovules. These results indicated that SUP requires features of the INO protein to repress transcription from PRO_{INO}. These features of INO are lacking in CRC. Coupled with evidence that showed that INO participates in positive feedback of its expression, it was concluded that SUP likely inhibits integument growth by disrupting INO autoregulation (Meister et al., 2002).

Following this study, Meister et al. (2005) tested the ability of other YABBY members to support outer integument growth. When PRO_{INO}:*YABBY3* (*YAB3*) was introduced into *ino-1* plants, ovules exhibited outer integument growth on all sides of the ovules. This indicated a poor response to SUP repression similar to the response to the presence of PRO_{INO}:*CRC*. In contrast, when PRO_{INO}:*YAB5* was introduced in *ino-1* plants, outer integument growth did not occur, and thus YAB5 was unable to support outer integument growth. To determine the differences between INO and other YABBY members that led to these results, domain swaps between regions of CRC and INO were created. By observing the ability of chimeric transgenes composed of regions 1, 2, or 3 and 4 collectively (Fig. 2A) of CRC or INO to support proper outer integument development, it was determined that multiple regions contributed to INO-specific function in a largely quantitative manner (Meister et al., 2005). While all CRC/INO domain swap lines were able to support outer integument growth, only a subset responded to SUP repression of growth on the gynoapical side of ovules. This suggested that all three regions of INO participate in both the promotion of growth and response to SUP inhibition, although the combination of regions 3 and 4 appeared to play a more significant role (Meister et al., 2005). These results, however, may have been specific to domain swaps between INO and CRC due to the unique role of CRC in gynoecium and nectary specification, in addition to its role in abaxial identity that is common among all YABBY proteins (Alvarez and Smyth, 1999). This is particularly of concern due to the possibility that INO and CRC regions may share some redundancy in function that is not present in other YABBY members, as INO and CRC are the only members with specific reproductive roles.

In our current study, exchanges between regions of INO and YAB3 were created to test the hypothesis that domain swap results with INO and CRC resulted from reproductive-specific features of CRC. To separate SUP-responsive defects from growth-promotive effects, domain swap experiments between INO and YAB5 were conducted in parallel, because PRO_{INO}:*YAB5* did not support outer integument growth in *ino-1* plants. To refine our understanding of the importance of the C terminus that was observed in Meister et al. (2005), more defined swaps were made to separate the YABBY region from the more C-terminal portion in domain swap experiments between INO and CRC and INO and YAB3. Through these studies, we show that multiple regions of INO contribute toward the promotion of growth and response to SUP; however, the mechanisms underlying each function are distinct in that a quantitative response was observed only for SUP-related effects.

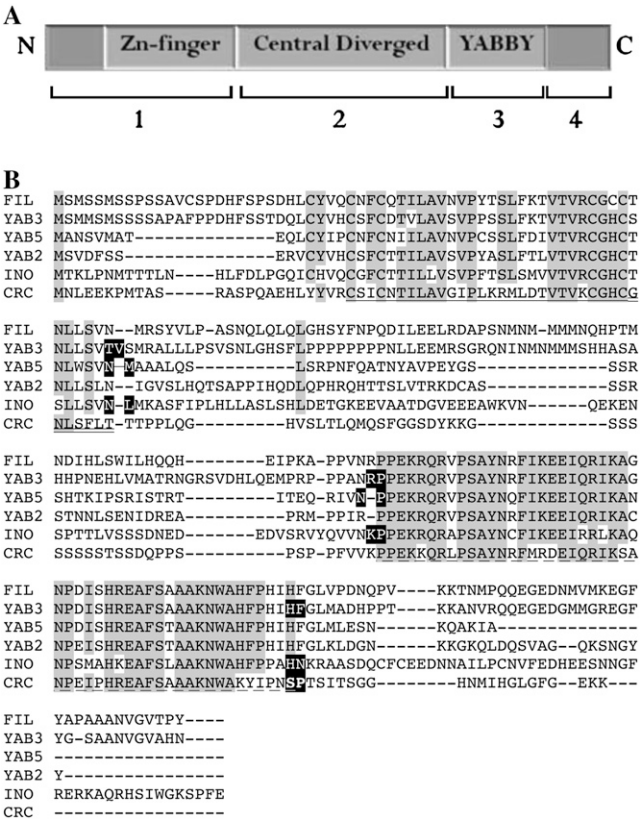

Figure 2. Features of the YABBY family of transcription factors. A, A cartoon representation of the four major regions exchanged in domain swap experiments. The regions designated by the numbers at bottom represent: 1, the N-terminal and Zn-finger regions; 2, the central variable region; 3, the YABBY region; and 4, the C-terminal region. B, An amino acid alignment of YABBY proteins. The Zn-finger and YABBY regions, the borders of which were determined based on previous sequence alignments (Siegfried et al., 1999), are indicated using single solid and broken underlines, respectively. The seven conserved amino acid residues adjacent to the previously defined Zn-finger motif are indicated with a double underline. Residues on either side of protein region boundaries that were used in assembly of chimeric proteins are highlighted in black.

RESULTS

Nomenclature

In the following sections, representation of the source of each of the exchanged regions in the chimeric cDNAs is indicated by the following: I indicates INO sequence, C indicates CRC sequence, 3 indicates YAB3 sequence, and 5 indicates YAB5 sequence. These designations are listed from left to right starting from the amino terminus and ending with the carboxy terminus. The majority of exchanges focused on three major regions of INO: the N-terminal and Zn-finger regions (1) comprised the first region, the central variable region (2) comprised the second region, and the YABBY (3) and C-terminal (4) regions together comprised the third region (Fig. 2A). However, since the third region possessed greater INO-specific function than the first and second regions in a prior study (Meister et al., 2005), we separately exchanged the YABBY (3) and C-terminal (4) regions in a subset of our experiments. Separate exchange of the C-terminal region 4 is indicated by the following: i indicates INO sequence, 3 indicates YAB3 sequence, and c indicates CRC sequence. Boundaries between regions were selected based on sequence conservation between YABBY members as well as results indicating the importance of seven conserved residues at the carboxyl end of the Zn-finger region (Fig. 2B; Siegfried et al., 1999; Villanueva et al., 1999; Bowman, 2000; Meister et al., 2005).

INO/YAB3 Chimera Expression Using PRO_{INO}

Chimeric cDNAs composed of *INO* and *YAB3* sequences were expressed using PRO_{INO}, and phenotypic effects on outer integument development were scored in an *ino-1* mutant background (Table I). Five phenotypic classes were observed among transformed lines containing chimeric transgenes: *sup*-like, weak-*sup*, wild type, weak-*ino*, and *ino*-like (Fig. 3). Individual transformants sometimes exhibited variation in outer integument development within gynoecia, and these lines were scored based on the phenotype of the majority of ovules of that line. The phenotypic classes observed were based on comparisons with the following control lines: *sup-5*, *ino-1*, *ino-1* with PRO_{INO}:*III* (*INO*), and wild type (Table I). In *sup-5* plants, ovules exhibited significant growth on both the gynobasal and gynoapical sides, while *ino-1* plants exhibited no growth of the outer integument. Most ovules from *ino-1* plants containing PRO_{INO}:*III* resembled wild type, indicating rescue of the *ino-1* phenotype.

To determine the significance of differences observed in ovule development for chimeric lines as compared to the control lines, the data were subjected to a Fisher's exact test. A Bonferroni adjustment was used to adjust the α value to account for the increased probability of error when numerous pairwise comparisons are made within a single set of data (Sonnenberg, 1985). Statistical analyses of all domain swap lines in comparison to control lines are reported in Supplemental Tables S1 to S6.

PRO_{INO}:*III* versus PRO_{INO}:*III/YAB3* Swaps

Ovules of transgenic lines expressing *3II*, *II3*, or *III3* under PRO_{INO} commonly exhibited wild-type growth of the outer integument and were not significantly different from ovules of lines containing PRO_{INO}:*III* (Table I). In contrast, transgenic lines *I3I*, *3I3*, *I33*, *33I*, and *333i* expressed under PRO_{INO} were significantly different from PRO_{INO}:*III* plants, with increasing significance as listed from left to right (Table I; Supplemental Table S1). Plants from these lines contained ovules that were more like those induced by PRO_{INO}:*333* whereby growth of the outer integument frequently occurred on both the gynobasal and the gynoapical sides of the ovules. Based on these results, it appears

Table I. *Transgenic complementation of ino-1*

Genotype[a]	PRO_{INO}:Transgene[b]	Ovule Phenotype[c]					Significantly Different from PRO_{INO}:[d]			
		sup-Like	Weak-*sup*	Wild Type	Weak-*ino*	*ino*-Like	III	333	555	CCC
Wild type		–[e]	–	25	–	–	No	Yes	Yes	Yes
ino-1 SUP		–	–	–	–	25	Yes	Yes	No	Yes
INO sup-5		24	–	–	–	–	Yes	Yes	Yes	Yes
	III	–	–	28	8	6	~[f]	Yes	Yes	Yes
	333	–	11	–	8	–	Yes	~	~	~
	555	–	–	–	3	18	Yes	~	~	~
	CCC	7	13	–	4	1	Yes	~	~	~
	I33	6	9	6	2	1	Yes	Yes	~	~
	I3I	1	8	10	7	1	Yes	Yes	~	~
	II3	3	1	13	3	1	No	Yes	~	~
	3II	–	1	20	2	–	No	Yes	~	~
	33I	–	14	5	1	–	Yes	Yes	~	~
	3I3	–	10	18	–	–	Yes	Yes	~	~
	333i	–	14	3	3	–	Yes	No	~	~
	III3	–	–	21	4	1	No	Yes	~	~
	I55	–	–	2	17	8	Yes	~	Yes	~
	I5I	–	–	3	22	3	Yes	~	Yes	~
	II5	1	3	9	9	4	No	~	Yes	~
	5II	–	–	3	20	3	Yes	~	Yes	~
	55I	–	–	6	14	7	Yes	~	Yes	~
	5I5	–	–	1	22	6	Yes	~	Yes	~
	IIIc	3	8	13	2	0	Yes	~	~	Yes
	CCCi	3	4	2	18	1	Yes	~	~	Yes

[a]Ovules were examined in wild-type, *ino-1*, or *sup-5* mutant plants. [b]Transgenes were constructed as transcriptional fusions of the listed coding sequence with PRO_{INO} and examined in an *ino-1* mutant background. [c]Number of transgenic lines with the corresponding ovule phenotype, as described in the text and illustrated in Figure 3. [d]Pairwise comparisons of the phenotypic class distribution for each line. [e]–, No plants of this class observed. [f]~, Pairwise comparison not applicable.

that the presence of two adjacent regions of INO is sufficient to promote outer integument development like that of the full-length INO protein, with no significant difference between II3 and 3II when compared to INO or each other (Tables I and II). Both I33 and I3I chimeras were significantly different from INO and 3II but were not significantly different from II3. This indicates that adjacent regions 2, 3, and 4 are better able to support growth like that of INO than adjacent regions 1, 2, and 3 (Fig. 2A). C-terminal domain swaps between YAB3 and INO show that the C terminus of INO is not essential for INO function in this context, because PRO_{INO}:*III3* transgenics were not significantly different from PRO_{INO}:*III* transgenics. Taken together, this indicates that the central variable and YABBY regions contain important information for the unique function of INO in responding to repression by SUP.

PRO_{INO}:*333* versus PRO_{INO}:*INO/YAB3* Swaps

When comparing PRO_{INO}:*INO/YAB3* domain swap lines with lines expressing PRO_{INO}:*333* (in an *ino-1* background), all lines except *I3I* and *333i* transgenics were significantly different from PRO_{INO}:*333* (Table I). Although INO/YAB3 domain swap lines were similar to PRO_{INO}:*333* transgenics (all swaps resulted in a proportion of individuals with weak-*sup* ovules), many domain swap lines possessed wild-type ovules, in contrast with PRO_{INO}:*333* transgenics. This is notable with PRO_{INO}:*I3I*, a line that did not meet our most stringent criterion for statistically significant difference from PRO_{INO}:*333*. However, this construct led to a total of 10 wild-type individuals, while PRO_{INO}:*333* did not result in any wild-type individuals and in fact was different from PRO_{INO}:*333* if the significance level was raised to a mere 1.04% cutoff. We therefore consider this line to show a real difference from PRO_{INO}:*333*. Although all lines were able to support growth of the outer integument, the extent of growth varied among lines (Table I). Based on our results, the central variable region of YAB3 contains sufficient ability to promote some growth of the outer integument on the gynoapical side of ovules, as evidenced by PRO_{INO}:*I33*, *I3I*, and *33I* transgenic lines. The presence of adjacent regions from *YAB3* did not enhance the ability of chimeric proteins to stimulate outer integument growth, because PRO_{INO}:*I33* and *33I* were not significantly different from PRO_{INO}:*I3I* (Table II). The results for PRO_{INO}:*333i* indicate that the C terminus of YAB3 is not necessary for the promotion of growth nor escape from SUP repression when expressed in ovules, as these transgenics were not significantly different from PRO_{INO}:*333* plants. This also shows that all three primary regions of INO contribute to its ability to respond to SUP, with the central variable region providing the smallest increment of this activity.

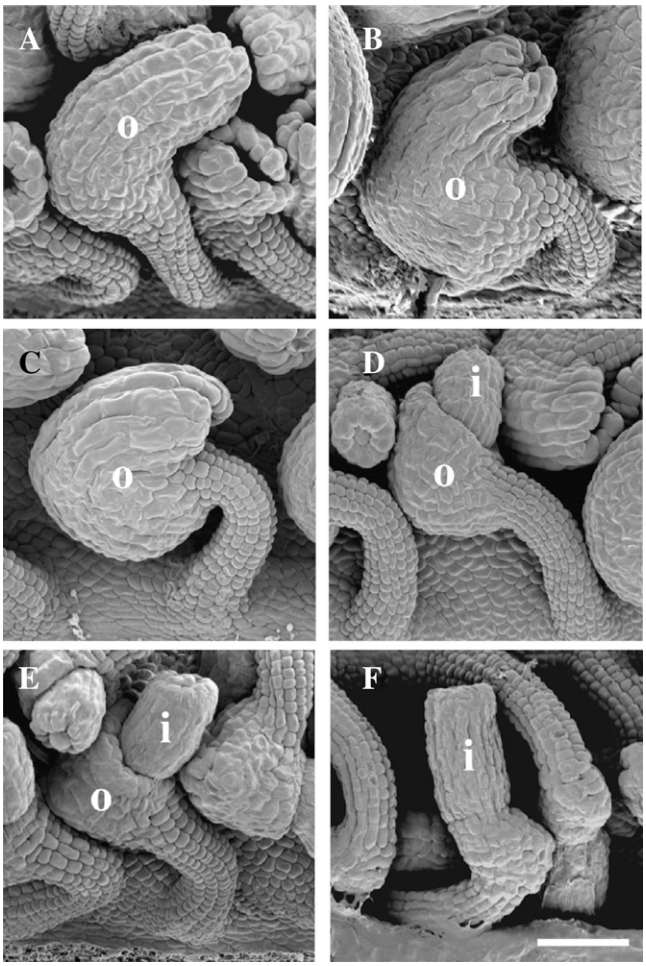

Figure 3. Scanning electron micrographs of representative ovule phenotypic classes observed from *INO* and *YAB3*, *YAB5*, or *CRC* chimeric cDNAs expressed using PRO_{INO} in an *ino-1* mutant background. Growth of the outer integument occurred on both the gynobasal and gynoapical sides of ovules of some transformants as shown in A and B, representing *sup*-like and weak-*sup* classes, respectively. Other lines showed a wild-type phenotype with growth initiating and proliferating primarily on the gynobasal side of the ovule (C). D and E, A range of outer integument growth was exhibited in lines classified as weak-*ino*. F, Some transgenic lines showed no outer integument growth and thus exhibited the unaltered *ino-1* mutant phenotype. i, Inner integument; o, outer integument. Scale bar = 50 μm.

INO/YAB3 Chimera Expression Using the Cauliflower Mosaic Virus 35S Promoter

In parallel with phenotypic analysis of chimeric YABBY expression using PRO_{INO}, ectopic expression using the generally active cauliflower mosaic virus 35S promoter ($PRO_{35SCaMV}$) was pursued to address the functionality of the chimeric cDNAs. When expressed ectopically, YABBY family members, including both *YAB3* and *INO*, have been shown to alter leaf morphology, leading to narrowed and curled leaves, likely due to the abaxialization of adaxial tissue types (Eshed et al., 1999; Siegfried et al., 1999; Meister et al., 2005). Detection of similar effects from ectopic expression of the chimeric YABBY/INO genes would show that the genes produced functional YABBY-type proteins. With examples shown in Figure 4, all chimeric lines created with the $PRO_{35SCaMV}$ expression vectors resulted in leaf abnormalities consistent with ectopic YABBY effects. Thus, all of the chimeric proteins functioned as YABBYs, as was observed in prior domain swap experiments with CRC and INO (Meister et al., 2005).

INO/YAB5 Chimera Expression Using PRO_{INO}

The majority of *ino-1* mutant plants harboring the PRO_{INO}:*555* (*YAB5*) construct exhibited no growth of the outer integument, and when any growth was observed, it was very limited (Table I). Following the strategy described for domain swaps between YAB3 and INO, chimeric YABBY proteins composed of YAB5 and INO were created and expressed using PRO_{INO}. As with the YAB3 and INO domain swaps, a range of phenotypes was observed and compared to the control lines (Fig. 3; Table I). The data were analyzed as for the YAB3/INO domain swaps.

PRO_{INO}:*III* versus PRO_{INO}:*INO/YAB5* Swaps

Although the ability to support outer integument growth was markedly less than that seen for INO/YAB3 domain swaps, all INO/YAB5 chimeras were able to support some degree of growth (Table I). The transgenic line PRO_{INO}:*II5* was the only line that did not significantly differ from PRO_{INO}:*III* (Table I), because a majority of ovules from this line exhibited wild-type growth of the outer integument. This line, however, was not significantly different from PRO_{INO}:*5II* or *55I* lines that most often resulted in ovules with weak *ino* phenotypes (Tables I and II), indicating that some degradation of activity relative to the wild-type INO protein is likely. Overall, the data demonstrate that any one region from INO is sufficient to promote outer integument growth, albeit weakly in many INO/YAB5 chimeric lines. A quantitative effect was not observed, as PRO_{INO}:*5II* did not support growth to a greater extent than PRO_{INO}:*55I*.

PRO_{INO}:*555* versus PRO_{INO}:*INO/YAB5* Swaps

All INO/YAB5 domain swap lines expressed using PRO_{INO} (in an *ino-1* background) were significantly different from *ino-1* plants expressing PRO_{INO}:*555* (Table I). This is largely because a YAB5/INO chimeric gene containing any one region of INO was sufficient to support some growth of the outer integument for all chimeric lines observed. As expected, *ino-1* plants were not significantly different from PRO_{INO}:*555* plants, because YAB5 was rarely able to support any growth of the outer integument (Table I).

INO-YAB5 Chimera Expression Using $PRO_{35SCaMV}$

As with ectopic expression analysis of YAB3 and INO domain swaps, all chimeric lines of domain

Table II. *Pairwise comparisons of phenotypic class distribution*

PRO_{INO}:Transgene Comparison[a]	*P* Value[b]	Significantly Different from Each Other
I33 versus 3II	2.31×10^{-05}	Yes
I33 versus 3I3	1.20×10^{-03}	Yes
I3I versus 3II	3.00×10^{-03}	Yes
I3I versus 3I3	6.20×10^{-03}	Yes
II3 versus 33I	5.33×10^{-05}	Yes
II3 versus 3I3	1.30×10^{-03}	Yes
3II versus 33I	7.84×10^{-06}	Yes
3II versus 3I3	5.00×10^{-03}	Yes
I33 versus I3I	1.02×10^{-01}	No
I33 versus II3	2.13×10^{-02}	No
I33 versus 33I	4.56×10^{-02}	No
I3I versus II3	6.83×10^{-02}	No
I3I versus 33I	3.77×10^{-02}	No
II3 versus 3II	1.71×10^{-01}	No
33I versus 3I3	1.16×10^{-02}	No
I55 versus II5	8.60×10^{-03}	Yes
I5I versus II5	5.90×10^{-03}	Yes
II5 versus 5I5	9.62×10^{-04}	Yes
I55 versus I5I	2.30×10^{-01}	No
I55 versus 5II	2.54×10^{-01}	No
I55 versus 55I	4.10×10^{-01}	No
I55 versus 5I5	5.73×10^{-01}	No
I5I versus 5II	1.00×10^{00}	No
I5I versus 55I	1.23×10^{-01}	No
I5I versus 5I5	4.02×10^{-01}	No
II5 versus 5II	1.14×10^{-02}	No
II5 versus 55I	1.55×10^{-01}	No
5II versus 55I	1.94×10^{-01}	No
5II versus 5I5	4.08×10^{-01}	No
55I versus 5I5	7.15×10^{-02}	No
IIIc versus CCCi	1.21×10^{-05}	Yes

[a]Transgenes were constructed as transcriptional fusions of the listed coding sequence with PRO_{INO} and examined in an *ino-1* mutant background. [b]The phenotypic class distribution of each set was calculated using Fisher's exact test to determine the *P* value.

swaps between YAB5 and INO resulted in the characteristic YABBY overexpression phenotypes observed previously (Fig. 4; Eshed et al., 1999; Siegfried et al., 1999; Meister et al., 2005). Thus, the weak or absent ability of the chimeric proteins to support integument growth results from specific properties of the proteins, rather than being an indication of a generally inactive protein.

C-Terminal Domain Swaps Using PRO_{INO}

As mentioned previously, domain swap experiments that exchanged regions 1, 2, or 3 and 4 from CRC and INO suggested that regions 3 and 4 provided more functional information than regions 1 and 2 (Fig. 2A; Meister et al., 2005). To address whether this specific information was contained within region 3 or 4, these regions were swapped to individually evaluate their roles. When the C-terminal domain swaps were expressed using PRO_{INO}, the majority of lines showed an ability to support outer integument growth (Fig. 3). The strategy for classification described above was used to evaluate phenotypes and statistical significance.

Transgenic lines expressing *IIIc* or *CCCi* under PRO_{INO} were significantly different from PRO_{INO}:*III*, with a larger degree of significance for the latter line (Table I; Supplemental Table S5). These results are due to a significant proportion of PRO_{INO}:*IIIc* plants that contained ovules with weak-*sup* or *sup*-like phenotypes. This suggests that the C terminus of CRC is sufficient to overcome the repressive action of SUP. In contrast, the majority of transgenic lines containing PRO_{INO}:*CCCi* were only able to support outer integument growth weakly (Table I). This result implies that the ability of CRC domain swaps to support outer integument growth like that of PRO_{INO}:*CCC* (*CRC*) transgenic plants was dependent on the presence of the C-terminal region of CRC. As expected, *IIIc* and *CCCi* were significantly different from each other (Table II) due to the ability of *IIIc* to produce wild-type ovules, whereas *CCCi* provided only limited support for growth of the outer integument.

PRO_{INO}:*IIIc* and *CCCi* transgenic lines were significantly different from PRO_{INO}:*CCC* plants (Table I). Although several lines with PRO_{INO}:*IIIc* had weak-*sup* and *sup*-like phenotypes, several plants also had wild-type ovules unlike plants containing PRO_{INO}:*CCC*. PRO_{INO}:*CCCi* plants rarely supported outer integument growth more than that observed for a weak-*ino* phenotype, indicating the importance of the C-terminal portion of CRC for CRC-like function when expressed in ovules.

C-Terminal Domain Swaps Using $PRO_{35SCaMV}$

As for the other tested constructs, the C-terminal domain swaps were tested for YABBY function through expression from $PRO_{35SCaMV}$. The resulting chimeric lines exhibited leaf abnormalities consistent with ectopic YABBY effects. Thus, the limited function of CCCi in supporting integument growth does not appear to result from a general loss of all protein activity.

DISCUSSION

Previous work using INO and CRC domain swaps by Meister et al. (2005) addressed the particular properties of these two reproductive-specific YABBY members in the context of growth promotion and response to SUP inhibition. The unique role of CRC during nectary development (Bowman and Smyth, 1999; Baum et al., 2001), however, indicates that the effects observed in INO/CRC domain swaps may have been due to features not shared by other YABBY members. Such a specialized divergence for CRC is consistent with the observation that other YABBY proteins cannot substitute for CRC in carpel or nectary development (Meister et al., 2005). To address this issue, our current study expands on this work by examining domain

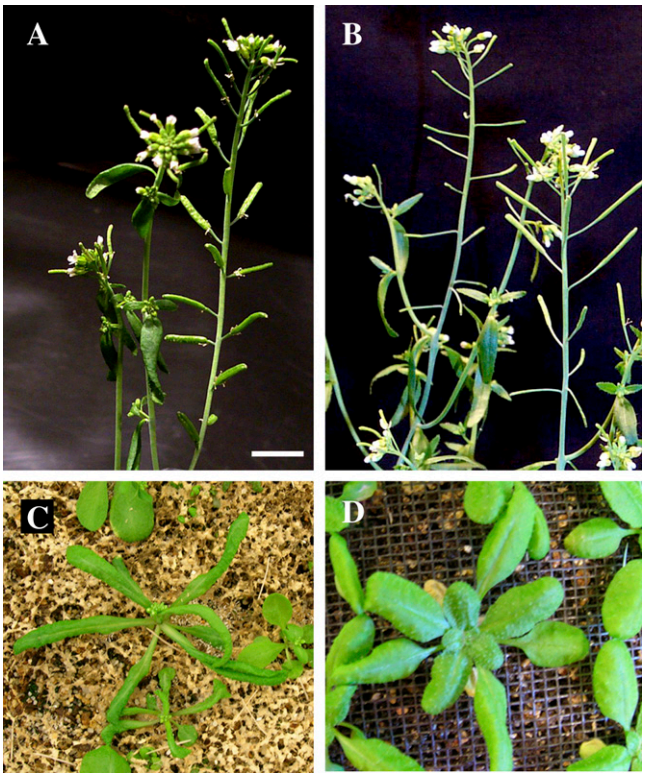

Figure 4. Ectopic expression of wild-type or chimeric YABBY proteins produced alterations in vegetative and reproductive development. Expression using $PRO_{35SCaMV}$ often resulted in gynoecia and cauline leaves that were misshapen and had irregular surfaces (A) when compared to wild-type Arabidopsis (B). Leaves of the transgenic plants were often narrow and epinastic (C) as compared to those of wild-type plants (D). Scale bar = 10 mm.

swaps between INO and YAB3, a YABBY protein produced in both vegetative and reproductive organs that also fails to respond to SUP, and YAB5, the only Arabidopsis YABBY member that failed to support outer integument growth when expressed under PRO_{INO} (Meister et al., 2005).

YAB3 and INO Domain Swaps

Our results using YAB3/INO domain swaps were largely similar to those for CRC and INO, indicating that the reproductive role of CRC does not confer a special ability to function in INO/CRC chimeras. In INO/YAB3 domain swap lines, the presence of adjacent regions 2, 3, and 4 from INO supported outer integument growth more like that of the complete INO protein than chimeras that possessed only one region from INO or two, nonadjacent regions from INO. This observation parallels that of Meister et al. (2005), who found that INO/CRC domain swap lines were most similar to wild type when regions toward the C-terminal portion of INO were included. Furthermore, it was found that inclusion of the central variable region of YAB3 was sufficient to overcome SUP repression in most cases. This suggests that there are responsive elements to SUP repression within the central variable region of INO that are lacking in YAB3, as the differential response to SUP is the only difference observed between the expression of *YAB3* or *INO* using PRO_{INO}.

Although the presence of the central variable region of YAB3 in I3I did confer some resistance to SUP repression, the central region of INO in 3I3 was not sufficient for the proper response to SUP repression (Table I). This implies that there are sequences outside of the central variable region that are also important for proper SUP repression. In contrast, it appears that there is little information necessary for INO function within the C terminus, because plants expressing INO with an exchanged C terminus (PRO_{INO}:*IIIc* and PRO_{INO}:*III3*) possessed wild-type ovules (Table I).

YAB5 and INO Domains Swaps

PRO_{INO}:*555* plants rarely supported any outer integument growth, but plants possessing YAB5/INO chimeras of any combination were capable of supporting outer integument growth at least to a limited extent (Table I). This indicates that all regions contribute to INO-specific function. While PRO_{INO}:*I15* resulted in ovule phenotypes that could not be statistically differentiated from PRO_{INO}:*III*, no other chimeric combinations between INO and YAB5 were able to support growth as well as INO itself. Interestingly, the other chimeric YAB5/INO transgenes with two regions from INO did not exhibit any greater rescue of the *ino-1* phenotype than did YAB5/INO chimeric proteins with only one region from INO. Therefore, a combination of differences between INO and YAB5 distributed over at least three regions of the protein is required to render the protein incapable of supporting outer integument growth in *ino-1* plants.

In contrast with domain swaps between INO and CRC or YAB3, quantitative effects due to increase in the fraction of INO content were not observed for YAB5/INO chimeras. Based on our hypothesis that INO makes multiple contacts with proteins and/or DNA targets to elicit INO-specific function, these results for YAB5/INO domain swaps were surprising. Although we do not present direct evidence, it seems likely that the YAB5 protein is capable of binding to INO DNA targets but fails to drive proper expression of these genes. This is supported by our observation that 5I5 chimeras, which contain DNA-binding domains exclusively of YAB5 origin, supported outer integument growth to a limited extent, although we cannot rule out the potential role of the central variable region in this interaction.

It is likely that the binding of YAB5 to INO DNA targets, or its interaction with trans-factors needed for expression of these targets, is inefficient due to the absence or misorientation of critical residues that facilitate these interactions. Reduction in these inter-

actions could be produced by disruption of intramolecular contacts necessary for formation of the most active protein structure due to the presence of the YAB5 sequences. If the presence of any region of YAB5 could cause such disruption, then this could explain the lack of additivity observed for multiple segments of INO. Thus, any level of contribution from INO to YAB5/INO chimeras promotes growth, but the ability to support growth like that of full-length INO requires interactions between all three regions of the chimeric protein. Alternatively, the nonquantitative behavior of INO in these chimeras may simply be a result of greater structural divergence of YAB5 from other YABBY members, disrupting intermolecular interactions no matter the amount of INO identity within each chimera. We hypothesize that YAB3 and CRC at least partially conserve the inter- and intramolecular contact sites and so behave more like INO regions in the chimeric proteins.

C-Terminal Domain Swaps

YAB3/INO C-terminal domain swaps had little effect on the ability of the chimeric proteins to influence ovule development (Table I). These results suggest that the C-terminal portion of INO does not include information that can overcome the *sup*-like response observed when the YAB3 protein is expressed in ovules.

In contrast with the C-terminal domain swaps between YAB3 and INO, the swaps between CRC and INO show that the C-terminal region of CRC does possess unique information (Table I). The distribution of phenotypes among transgenics with CCCi spans the entire range of phenotypic classes; however, the majority of plants possessed weak-*ino* ovules. This suggests that the ability of the CRC protein to both support outer integument growth and overcome SUP repression is at least partially dependent on certain residues contained within the C-terminal region. This could reflect the need for specific intermolecular interactions between the C-terminal region and the rest of the CRC protein for formation of an active structure. In further support of the importance of the C-terminal region, while most plants containing PRO_{INO}:*IIIc* exhibited a wild-type ovule phenotype, several transgenic plants possessed weak-*sup* ovules and *sup*-like ovules (Table I). Thus, the presence of the C-terminal portion of CRC was sufficient for the IIIc protein to overcome SUP repression in some plants. This ability, however, is not exclusively found within the C-terminal region, as PRO_{INO}:*IIIc* plants did not support the *sup*-like or weak-*sup* phenotype as frequently as PRO_{INO}:*CCC*. Based on the results of YAB3/INO domain swaps, it appears unlikely that the C-terminal portion of INO played a role in the phenotypes observed for PRO_{INO}:*IIIc* and PRO_{INO}:*CCCi*; rather, the presence or lack of the C-terminal region of CRC was the critical factor in determining the extent and nature of outer integument growth.

CONCLUSION

We have found that no particular region of INO contained the specific information responsible for either the differential promotion of outer integument growth or differential response to SUP. This parallels results described for INO/CRC domain swaps in Meister et al. (2005), indicating that reproductive-specific features of CRC do not contribute to the effects observed for INO/CRC domains swaps. Furthermore, we present novel evidence that shows that the C-terminal region of CRC is necessary for chimeric proteins to support outer integument growth and escape SUP suppression in ovules, while, in contrast, the C-terminal region of INO does not appear to possess unique information necessary for INO function. We have further shown that while the central variable region of YAB3 is sufficient to overcome SUP growth repression, the central variable region of INO is not sufficient to mediate suppression by SUP. This novel finding suggests that while there are SUP-responsive elements in the central variable region of INO that have diverged in YAB3, there exist elements outside of the central variable region of INO that participate in the response to SUP. Lastly, we have shown that a single region derived from INO is sufficient to confer growth-promoting activity of YAB5/INO chimeras; however, in contrast with YAB3 and CRC domains swaps with INO, features of INO are required along the entire length of YAB5/INO chimeras for wild-type outer integument growth.

The positive quantitative effect of INO observed in domain swaps with YAB3 and CRC may reflect multiple unique adaptations of the INO protein for the specialized role it plays in reproductive development. Such specialization is supported by the observation that YAB5 cannot substitute for INO but ectopic expression of YAB5 or INO results in similar vegetative phenotypes. Thus, features of YABBY proteins necessary for interactions that lead to the vegetative effects of ectopic expression appear to be conserved among all Arabidopsis YABBY paralogs, and intramolecular interactions necessary for establishing a structure that can function in this capacity must also be preserved.

From a mechanistic standpoint, our data indicate that the INO protein makes multiple contacts with proteins or DNA sequences that are required for the proper temporal and spatial expression of genes necessary for the establishment of the outer integument. Different sets of intermolecular contacts may be disrupted by particular differences between INO, CRC, YAB3, and YAB5, explaining why different regions of these proteins have the greatest effects in domain swaps. In support of this observation, in vitro studies using pull-down assays demonstrated an ability of the Zn-finger and YABBY regions of the INO protein to independently bind the transcription factor NOZZLE/SPOROCYTELESS, a protein that is necessary for the establishment of the nucellus and pollen sacs in Arabidopsis (Sieber et al., 2004). This supports our findings that domains with discrete function cannot be

readily identified in INO and that the protein instead acts as an integrated unit that likely requires multiple intermolecular interactions on a variety of surfaces of INO to form active complexes capable of driving proper outer integument growth.

Overall, our results suggest that INO's function during ovule development involves a coordination of molecular events that are distinct from those events that are shared among the YABBY family. These reproductive-specific events, dependent upon all three regions of INO, are necessary for the promotion of integument growth and the response to modulation by SUP. While YAB3 and CRC can participate in all the intermolecular interactions necessary for the promotion of growth, they fail to participate in interactions necessary for repression by SUP. Biochemical identification of factors that associate with INO and gene targets of INO-mediated transcriptional regulation will elucidate the mechanisms by which INO promotes the development of the outer integument and reveal the particular contacts made by each region of the protein. Because we have not detected physical interactions between INO and SUP, or INO and PRO_{INO} using yeast two- and one-hybrid studies, respectively (data not shown), the identification of additional factors involved in this process is key to understanding the molecular events that coordinate ovule development.

MATERIALS AND METHODS

Construct Assembly

Chimeric Coding Sequences

Chimeric coding sequences were created using overlap extension PCR (Horton et al., 1990) to create fusions between regions of YAB3, YAB5, INO, and CRC. Using the same strategy employed by Meister et al. (2005), chimeric cDNAs were synthesized and inserted into pLITMUS28 (New England Biolabs) using the restriction sites *Bam*HI/*Xba*I or *Eco*RI/*Xba*I depending on the source of template being used (data not shown). The sequence of the chimeric cDNAs was confirmed on an Applied Biosystems 3730 sequencing system.

PRO_{INO} Expression Constructs of Chimeric cDNAs

Chimeric cDNAs were introduced into pRJM42 that contained the regions 5′ and 3′ of the genomic INO coding sequence necessary for the proper temporal and spatial expression pattern of endogenous *INO* (Meister et al., 2004). Assembly of these constructs was performed using the restriction sites *Bam*HI/*Xba*I or *Bgl*II/*Xba*I according to the particular chimeric cDNA used (data not shown). This cloning strategy replaced the CRC coding sequence originally incorporated in pRJM42.

$PRO_{35SCaMV}$ Expression Constructs of Chimeric cDNAs

Chimeric cDNAs digested with *Bgl*II/*Xba*I were introduced into pMON999 digested with the same enzymes. pMON999 contained a modified $PRO_{35SCaMV}$ (Kay et al., 1987) with a polyadenylation signal sequence of nopaline synthase 3′.

Plant Growth and Transformation

Fragments including the chimeric cDNAs in the appropriate expression cassettes listed above were excised with *Not*I, the chimeric genes were inserted into pMLBART using the same site, and the resulting plasmids were transferred into the *Agrobacterium* strain ASE via triparental matings (Figurski and Helinski, 1979; Fraley et al., 1985; Gleave, 1992). Wild-type Landsberg *erecta* (L*er*) and *INO* (Columbia/*ino-1* [L*er*]) plants (Arabidopsis [*Arabidopsis thaliana*]) were grown on soil in constant light at 20°C for transformation using the floral dip method (Kranz and Kirchheim, 1987; Clough and Bent, 1998). T1 lines were selected following germination on soil by treatment with the herbicide glufosinate ammonium (Finale; Farnham Companies), which selects for the phosphinothricin acetyl transferase gene. Homozygous *ino-1* plants were identified using a detectable polymorphism between Columbia and L*er* ecotypes at the *INO* locus as described previously (Meister et al., 2002).

Microscopy

Determination of phenotype class for transformants containing chimeric cDNAs expressed using PRO_{INO} was initially performed using dark-field microscopy. Representative lines of each phenotypic class were then fixed and prepared for analysis using scanning electron microscopy following methods described previously (Broadhvest et al., 2000).

Statistical Analysis

Statistical analysis of significant differences between data sets of each chimera tested and against control lines was performed using Fisher's exact test at the UC Davis Statistical Laboratory. Significant differences between phenotypic effects of the transgenes were calculated with the modified Bonferroni adjustment (α value/number of pairwise comparisons) to reduce the rate of false-positives while conducting simultaneous pairwise comparisons. This method has been shown to be advantageous when multiple statistical analyses are conducted simultaneously (Wright, 1992; Morikawa et al., 1996).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Pairwise comparison of ovule phenotypes between PRO_{INO}:*INO/YAB3* and PRO_{INO}:*III*.

Supplemental Table S2. Pairwise comparison of ovule phenotypes between PRO_{INO}:*INO/YAB3* and PRO_{INO}:*333*.

Supplemental Table S3. Pairwise comparison of ovule phenotypes between PRO_{INO}:*INO/YAB5* and PRO_{INO}:*III*.

Supplemental Table S4. Pairwise comparison of ovule phenotypes between PRO_{INO}:*INO/YAB5* and PRO_{INO}:*555*.

Supplemental Table S5. Pairwise comparison of ovule phenotypes between PRO_{INO}:*INO/CCC* and PRO_{INO}:*III*.

Supplemental Table S6. Pairwise comparison of ovule phenotypes between PRO_{INO}:*INO/CCC* and PRO_{INO}:*CCC*.

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