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Integument Development in *Arabidopsis* Depends on Interaction of YABBY Protein INNER NO OUTER with Coactivators and Corepressors

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ABSTRACT *Arabidopsis thaliana* INNER NO OUTER (INO) is a YABBY protein that is essential for the initiation and development of the outer integument of ovules. Other YABBY proteins have been shown to be involved in both negative and positive regulation of expression of putative target genes. YABBY proteins have also been shown to interact with the corepressor LEUNIG (LUG) in several systems. In support of a repressive role for INO, we confirm that INO interacts with LUG and also find that INO directly interacts with SEUSS (SEU), a known corepressive partner of LUG. Further, we find that INO can directly interact with ADA2b/PROPORZ1 (PRZ1), a transcriptional coactivator that is known to interact with the histone acetyltransferase GENERAL CONTROL NONREPRESSIBLE PROTEIN 5 (GCN5, also known as HAG1). Mutations in *LUG*, *SEU*, and *ADA2b/PRZ1* all lead to pleiotropic effects including a deficiency in the extension of the outer integument. Additive and synergistic effects of *ada2b/prz1* and *lug* mutations on outer integument formation indicate that these two genes function independently to promote outer integument growth. The *ino* mutation is epistatic to both *lug* and *ada2b/prz1* in the outer integument, and all three proteins are present in the nuclei of a common set of outer integument cells. This is consistent with a model where INO utilizes these coregulator proteins to activate and repress separate sets of target genes. Other *Arabidopsis* YABBY proteins were shown to also form complexes with ADA2b/PRZ1, and have been previously shown to interact with SEU and LUG. Thus, interaction with these corepressors and coactivator may represent a general mechanism to explain the positive and negative activities of YABBY proteins in transcriptional regulation. The LUG, SEU, and ADA2b/PRZ1 proteins would also separately be recruited to targets of other transcription factors, consistent with their roles as general coregulators, explaining the pleiotropic effects not associated with YABBY function.

KEYWORDS ovule; integument; polarity; YABBY gene; ADA2b

OVULES are the plant reproductive organs that develop into seeds. In *Arabidopsis*, ovule primordia form from the regions near the carpel margins. Each primordium initiates two lateral sheathing organs, the integuments, which arise independently from the central region (chalaza) along the proximal–distal axis. The inner integument grows as a radially symmetrical structure to envelop the terminal nucellus. The outer integument grows asymmetrically, initiating and growing more extensively on the gynobasal side of the ovule, extending to envelop both the inner integument and nucellus, and resulting in an amphitropous (recurved) shape at anthesis (Robinson-Beers *et al.* 1992; Schneitz *et al.* 1995). The integuments enable the formation of the female gametophyte (Skinner *et al.* 2001) and form the seed coat after fertilization.

Several genes that regulate integument growth in *Arabidopsis* have been identified [reviewed in Skinner *et al.* (2004) and Colombo *et al.* (2008)]. *INNER NO OUTER* (*INO*) is one such gene that encodes a YABBY protein, and loss of *INO* function results in the absence of the outer integument and disruption of gametophyte development in the ovule (Baker

et al. 1997; Schneitz et al. 1997; Villanueva et al. 1999). *INO* is the only YABBY gene expressed in the ovule, and expression is directly associated with outer integument growth being only in the abaxial layer on the side of the outer integument with maximal growth (Bowman and Smyth 1999; Sawa et al. 1999; Siegfried et al. 1999; Villanueva et al. 1999). Misexpression of *INO* on the opposite side of the ovule, as is observed in *superman* mutants, results in growth on both sides of the outer integument forming a more radially symmetrical structure (Gaiser et al. 1995; Meister et al. 2002). This indicates that, in the context of the chalaza, *INO* is sufficient for outer integument initiation and growth. Conservation of the specific expression pattern and function of *INO* orthologs in the outer integument has been observed in all other angiosperm species where it has been examined (Villanueva et al. 1999; Yamada et al. 2003, 2011; McAbee et al. 2005; Lora et al. 2011; Skinner et al. 2016).

The YABBY (YAB) gene family in *Arabidopsis* includes reproductively-expressed *CRABS CLAW* (*CRC*) and *INO*, and also *FILAMENTOUS FLOWER* (*FIL*), *YAB2*, *YAB3*, and *YAB5*, which exhibit both vegetative and reproductive expression (Villanueva et al. 1999; Bowman 2000). YABBY proteins regulate organ polarity and laminar expansion and include two conserved motifs: an amino-terminal region including a Cys_2 Cys_2 zinc finger, and a carboxyl-terminal YABBY region with similarity to the DNA-binding domains of high mobility group (HMG) transcription factors (Bowman and Smyth 1999; Sawa et al. 1999; Siegfried et al. 1999; Villanueva et al. 1999; Kanaya et al. 2001). While sequence-specific DNA binding of YABBY proteins was not found in initial direct binding assays (Kanaya et al. 2002), evidence of their association with DNA has been found by chromatin immunoprecipitation (ChIP) (Shamimuzzaman and Vodkin 2013) and protein binding to DNA microarrays (Franco-Zorrilla et al. 2014). The mechanisms by which INO and other YABBY proteins regulate transcription to affect development are not well understood. *FIL* was shown to both activate and repress putative target genes, and to activate gene expression in yeast, and evidence for similar functions was found for *YAB3* (Bonaccorso et al. 2012). A repressive role for YABBY proteins is also supported by the observation that YABBY proteins in *Antirrhinum majus* (including the *An. majus* ortholog of INO) can interact with the corepressor protein STYLOSA, an ortholog of the *Arabidopsis* LEUNIG (LUG) protein (Navarro et al. 2004; Sridhar et al. 2004). Similar interactions between YABBY proteins and LUG were also found in *Arabidopsis*, as were interactions with the related LEUNIG_HOMOLOG (LUH) protein (Stahle et al. 2009). YABBY proteins were also found to interact with SEUSS (SEU), a corepressor known to also partner with LUG (Franks et al. 2002; Navarro et al. 2004; Sridhar et al. 2004), and similar interactions were seen with the related SEUSS LIKE 1 (SLK1), SLK2, and SLK3 proteins (Stahle et al. 2009). Neither LUG nor SEU contain DNA-binding motifs; rather, these proteins are hypothesized to repress target gene expression through specific targeting by DNA-binding transcription factors (Conner and Liu 2000; Franks et al. 2002; Sridhar et al. 2004), and through

interaction with histone deacetylase and components of the mediator complex (Gonzalez et al. 2007). Genetic analyses show that *LUG*, *SEU*, and *SLK* genes affect multiple aspects of plant development, including contributing to the growth of the outer integument (Roe et al. 1997; Franks et al. 2002; Bao et al. 2010).

Herein we confirm that, as for other YABBY proteins, INO interacts with both LUG and SEU. In addition to interaction with these corepressors, we find a direct interaction between INO and other YABBY proteins and a transcriptional coactivator protein, ADA2b [also known as PROPORZ1 (PRZ1)]. ADA2 proteins, originally characterized in yeast and also examined in *Arabidopsis*, form multi-protein complexes and directly interact with the histone acetyltransferase (HAT) GENERAL CONTROL NONREPRESSIBLE PROTEIN 5 (GCN5) (Grant et al. 1997; Mao et al. 2006). As seen for LUG and SEU, we find that ADA2b also supports outer integument extension. Through genetic analysis, we find that *ADA2b* and *LUG* function independently to promote outer integument growth, consistent with a bifunctional nature for INO as both a repressor and activator of target genes. Together, these results show that interaction with these corepressors and coactivator may represent a general mechanism to explain the positive and negative activities of YABBY proteins in transcriptional regulation.

Materials and Methods

Vector construction

PCR was used to add *Bam*HI and *Xba*I sites 11 bp upstream and 160 bp downstream of the INO coding region (Villanueva et al. 1999), respectively, and the resulting fragment was cloned into pLITMUS28 (New England Biolabs, Beverly, MA) at these same sites, resulting in pRJM264. The GAL4BD-INO fusion was constructed by transferring the INO cDNA from pRJM264 to pGBKT7 (Clontech) using *Bam*HI and *Xba*I, creating pTLG176. An N-terminal truncation of INO was created by PCR using the primers INOYabBam5′ and INOYabPst3′ with pRJM264 as a template, and the resulting product was cloned into pLIMUS28 to create pTLG134, and then transferred to pGBKT7 using *Bam*HI/*Pst*I to create pTLG178. A C-terminal truncation of INO was created by PCR using the primers INO-5′A2 and INOZnPst3′ using pRJM264 as a template, and the resulting product was cloned to pLITMUS28 to create pTLG133, and then transferred to pGBKT7 using *Bam*HI/*Pst*I to create pTLG177.

The GAL4BD-FIL fusion was constructed by adding *Eco*RI and *Bam*HI sites to the FIL cDNA from Bowman and Smyth (1999) and inserting it at these sites into pGBKT7, creating pKLP90. GAL4BD-YAB2 and YAB5 fusions were constructed by transferring PCR-amplified cDNAs using primers NcoYAB2cDNA5′ and YABb2cDNA3′2, and NcoYAB5cDNA5′ and YAB5cDNA3′, with cDNA clones of these two proteins, and inserting the coding regions into pGBKT7 using *Nco*I/*Eco*RI, to create pKLP94 and pKLP89, respectively. GAL4BD-YAB3 and CRC fusions were constructed by transferring PCR-amplified cDNAs using primers YAB3cDNAf and YAB3cDNAr, and CRCcDNAf and CRCcDNAr, with cDNA clones of YAB3

and CRC, and inserting the sequences into pGBKT7 using *Eco*RI/*Pst*I to create pMKS131 and pMKS130, respectively.

A full-length cDNA clone of ADA2a was cloned into pCR4-TOPO (Invitrogen, Carlsbad, CA) by PCR amplification using the primers ADA2aNcoIf and ADA2aXhoIr and the plasmid U09217 from the *Arabidopsis* Biological Resource Center at Ohio State (GenBank ID AY040045) as a template to create pELG03. GAL4AD-ADA2a was created by transferring the ADA2a cDNA in pELG03 to pGADT7 using *Nco*I/*Xho*I to create pKLP71.

A full-length cDNA clone of ADA2b was cloned into the pGADT7 yeast two-hybrid vector by PCR amplification using the primers 5′ADA2b and 3′ADA2b using the clone U13318 from ABRC (GenBank ID AY143911) as a template, and then cloned to pGAD424 (Clontech) using the restriction enzymes *Bam*HI/*Sal*I to create pTLG179. TGAL4AD-ADA2b was created by transferring the ADA2b cDNA in pTLG179 *Nco*I/*Sal*I to pGADT7 *Nco*I/*Xho*I to create pKLP131.

A full-length cDNA clone of LUG (Conner and Liu 2000) was used as a template for PCR with the primers LUGf and LUG2r, the resulting product was cloned to pJET1.2 (Fermentas) to create pMKS144, and then cloned into pENTR4 (Invitrogen) using *Nco*I/*Xho*I to create pMKS146. The insert in pMKS146 was then transferred to pDEST-GADT7 (Rossignol *et al.* 2007) using LR clonase (Invitrogen) to create pMKS227, a GAL4AD-LUG fusion.

A full-length cDNA clone of SEU, pCRII-HFFL7 (Sridhar *et al.* 2004), was transferred to pLITMUS28 using *Hin*dIII/*Pst*I to create pMKS197, and subsequently transferred to pENTR4 using *Nco*I/*Xho*I to create pMKS198. The insert in pMKS198 was then transferred to pDEST-GADT7 using LR clonase to create pMKS203, a GAL4AD-SEU fusion.

Yeast two-hybrid assays

Yeast transformation, mating, and cDNA library screening were performed as described with a previously described *Arabidopsis* pistil cDNA library (Kelley *et al.* 2012). For the initial screen, pTLG176 (BD-INO) was transformed into Y187 (Clontech) and then mated with the cDNA library. Mating mixtures were screened on yeast media lacking leucine, tryptophan, adenine, and histidine. Plasmids were recovered from yeast and sequenced. Pairwise interaction tests were performed by directly transforming plasmids into AH109 (Clontech), and colonies were restreaked on selective media at least twice for verification. Quantitative ortho-nitrophenyl-β-D-galactopyranoside assays to measure β-galactosidase activity as an indication of protein–protein interactions in yeast were performed according to the manufacturer's instructions (Yeast Protocols Handbook, Clontech)

Plant material

Arabidopsis plants were grown under long-day conditions as previously described (McAbee *et al.* 2005). The alleles used in this study were *ada2a* (SALK_150349), *ada2b-1* (Vlachonasios *et al.* 2003), *ino-1* (Villanueva *et al.* 1999), *lug-1* (Liu and Meyerowitz 1995), and *seu-1* (Franks *et al.* 2002). All plants were genotyped using PCR-based markers to confirm the

presence of each mutation and the primers used are described in Supplemental Material, Table S1. The P-PRZ1::PRZ1:GFP (P-ADA2b::ADA2b:GFP) transgenic line (Anzola *et al.* 2010) was a kind gift from Christian Luschnig [University of Natural Resources and Applied Life Sciences (BOKU), Vienna, Austria].

Microscopy

Scanning electron microscopy and ovule clearings were performed as described (Kelley *et al.* 2009). Differential interference contrast light microscopy was performed as described (Meister *et al.* 2002). Confocal images were taken with a Zeiss LSM 710 system using 488 nm laser excitation and recording 493–598 nm emission wavelengths (Zeiss [Carl Zeiss], Thornwood, NY). Images were edited in Adobe Photoshop CS5.

Data availability

Plant materials are available from the cited sources from which they were obtained. Plasmids are available from the corresponding author on request.

Results

Identifying INO-interacting proteins

We performed a yeast two-hybrid screen to identify putative INO-interacting proteins, using the full-length INO protein fused to the GAL4 DNA-binding domain (BD-INO) as bait and a library of *Arabidopsis* pistil cDNA fused to the coding region of the GAL4 activation domain to produce prey. Several positive clones were identified, including a clone encoding the N-terminal region of the ADA2a transcriptional coactivator protein (Stockinger *et al.* 2001) and one encoding the C-terminal 85% of the LUG transcription corepressor protein (Conner and Liu 2000). Because these were both consistent with the hypothesized action of INO as a transcription factor, they were selected for further analysis.

INO interacts with ADA2a and ADA2b in yeast

We produced fusions of full-length ADA2a, and the related paralog ADA2b (PRZ1), and showed that both of these proteins interact with INO in yeast (Figure 1). We examined the interaction of the ADA2 proteins with two conserved domains of the INO protein: the N-terminal region including the zinc finger motif (INOΔC) and the C-terminal region including the YABBY domain (INOΔN) (Bowman and Smyth 1999; Villanueva *et al.* 1999). Both ADA2a and ADA2b proteins showed interaction with the C-terminal region of INO that includes the YABBY domain in a plating assay (Figure 1I). This interaction was confirmed for ADA2b with enzyme activity assays (Figure S1 in File S1). Both ADA2a and ADA2b proteins also displayed a statistically significant interaction with the N-terminal region of INO (INOΔC) in the enzyme activity assay (Figure S1 in File S1), but this interaction was less apparent in the plating assay (Figure 1F). Thus, the C-terminal region of INO may be the primary determinant
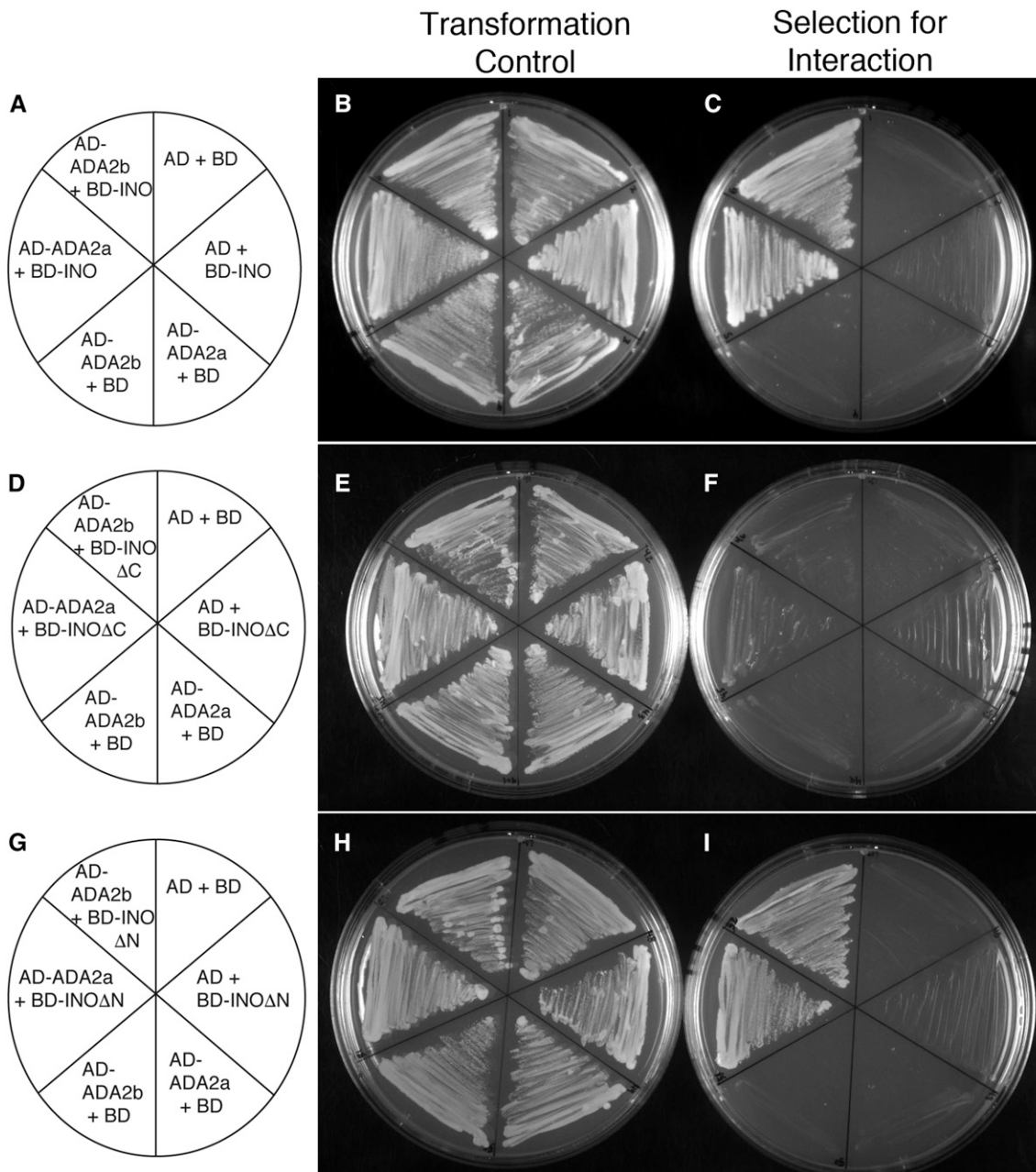
Figure 1 Yeast two-hybrid assays detect interactions between ADA2a and ADA2b and INO. (A, D, and G) Maps showing which proteins were produced in yeast strains streaked on the adjacent plates. "AD" and "BD" indicate the activation and DNA-binding domains of yeast GAL4, respectively. (B, E, and H) Streaks of indicated yeast strains on media lacking leucine and tryptophan, selecting only for the presence of the two expression plasmids in each strain. (C, F, and I) Streaks of indicated yeast strains on media lacking leucine, tryptophan, adenine, and histidine selected for interaction of the indicated proteins. The results show that none of the tested constructs significantly auto-activated, but that both AD-ADA2a and AD-ADA2b productively interact with both BD-INO and BD-INOΔN. A possible weak interaction of the ADA2 fusions with BD-INOΔC is also visible.

of interaction with these coactivators, but there may also be some contribution from the N-terminal region.

The interaction of ADA2 proteins with the conserved YABBY region of INO suggested that this interaction may be a general feature of YABBY proteins, not an exclusive interaction with INO. Yeast two-hybrid assays with the other *Arabidopsis* YABBY proteins revealed interactions of both ADA2a and ADA2b with FIL, YAB2, YAB3, and YAB5, but no interactions were observed with CRC (Figure S2 in File

S1 and Table 1). As previously reported (Bonaccorso *et al.* 2012), the BD-FIL fusion autoactivates reporter gene expression in yeast; however, we found that the growth of yeast (as a measure of protein interaction to activate selectable gene expression) is strongly enhanced when an ADA2 protein is also present (Figure S2 in File S1 and Table 1). Thus, our yeast two-hybrid data indicate that the ADA2 coactivator proteins can interact with all *Arabidopsis* YABBY proteins excepting CRC. The absence of observed phenotypic effects

Table 1 Yeast two-hybrid analysis of ADA2 interactions with YABBY proteins

BD	AD		
	Empty	ADA2A	ADA2B
Empty	—	—	—
FIL	+	+++	+++
YAB2	—	+	++
YAB3	—	+++	+++
YAB5	—	+	++
CRC	—	—	—
INO	—	+++	+++

AD, activation domain; BD, DNA-binding domain.

of loss-of-function mutations in *ADA2a* [Vlachonasios *et al.* (2003) and see below] led us to focus our further efforts on *ADA2b*.

For the interaction of ADA2b and INO to be biologically relevant, the two proteins must be present in the same cells and cellular compartment in the plant. We evaluated the expression pattern and cellular localization of ADA2b in ovules by examining the ovules of a line containing a reporter gene comprising the entire genomic copy of *ADA2b* with an in-frame fusion to a GFP coding region (Anzola *et al.* 2010). We found the transgene to be expressed in all cells of the ovule throughout development, with the fusion protein product being confined to the nucleus (Figure 2). INO is expressed in cells of the outer layer of the outer integument during the development of this organ, where it is also confined to the nucleus (Meister *et al.* 2002). Thus, the two proteins are present in the same cellular compartment in cells of the outer integument.

Loss of ADA2b function results in defective ovule development

Previously, the *ADA2b* loss-of-function allele, *ada2b-1*, was shown to have pleiotropic effects on plant growth and development, while loss of *ADA2a* did not have visible effects (Vlachonasios *et al.* 2003; Hark *et al.* 2009). Consistent with these previous reports, we found that *ada2a* mutants did not exhibit observable defects in vegetative or reproductive parts, including ovules (data not shown). In the *ada2b-1* mutant, in addition to the previously described vegetative defects, we observed defects in ovule development. Initiation and extension of the integuments from the chalaza through stage 2-V [stages according to Schneitz *et al.* (1995)] were unaffected and appeared similar to wild-type (Figure 3, A and E). However, at later stages of ovule development, both the outer and inner integuments exhibited growth defects. Growth of the outer integument was variable, but always reduced compared to wild-type, resulting in an exposed inner integument and nucellus (Figure 3, F–H). The growth of the outer integument was asymmetric, as in wild-type, resulting in a recurved shape of the ovule at anthesis (Figure 3, F and H). Growth of the inner integument was also reduced, and the nucellus was exposed beyond the inner integument at anthesis (Figure 3, F–H). We observed that inconsistent growth in

the margin of the inner integument produced gaps in this structure, and saw that the funiculus was increased in diameter in some ovules (Figure 3G).

The *ino-1* mutation eliminates the outer integument but the remainder of the ovule, including the inner integument, appears to continue to develop normally (Baker *et al.* 1997; Villanueva *et al.* 1999) (Figure 4, A and B). Consistent with a combination of the effects of the two mutations, the *ino-1 ada2b-1* double mutant lacked an outer integument and exhibited reduced inner integument growth (Figure 4, C and D). Gaps in the margin of the inner integument similar to those seen in *ada2b-1* were also present (Figure 4D). The double mutant ovules also lacked the outgrowth present on the gynoapical side (toward the apex of the gynoecium) of the chalaza that is present on *ino-1* mutant ovules (*cf.* Figure 4, B and D). The double mutant ovules varied in the visibility of the groove delineating the base of the inner integument with many lacking this feature, and cells in the funiculus became progressively less organized relative to wild-type (Figure 4, C and D). Thus, *ino-1* was epistatic to *ada2b-1* in promotion of outer integument growth, but *ada2b-1* had additional effects on ovule development in the inner integument, chalazal region, and funiculus, structures where *INO* is not expressed and does not play a role.

INO interacts with INO, LUG, and SEU in yeast

It was previously reported that some YABBY proteins were able to form homodimers and heterodimers (Stahle *et al.* 2009). Evaluation of INO protein in the yeast two-hybrid assay showed formation of an INO homodimer in yeast (Figure S3 in File S1 and Table 2). The potential interaction of INO with other YABBY proteins was not investigated because *INO* is the only YABBY gene expressed in the ovule (Bowman and Smyth 1999; Siegfried *et al.* 1999; Villanueva *et al.* 1999). Assays with truncated forms of INO showed that the C-terminal region (INOΔN), including the conserved YABBY domain but not the zinc finger region, was sufficient to promote the self-interaction of the INO protein in yeast (Figure S3 in File S1 and Table 2).

The yeast two-hybrid screen with BD-INO also identified a positive interaction with a truncated LUG protein. We found that INO also interacted with the full-length LUG protein (Figure S3 in File S1 and Table 2), as previously seen for FIL/YAB2/3 vegetatively expressed *Arabidopsis* YABBY proteins (Stahle *et al.* 2009). Interaction of these YABBY proteins with the LUG partner corepressor SEU has also been demonstrated (Stahle *et al.* 2009), and we found that INO also interacted with SEU in the yeast two-hybrid assay (Figure S3 in File S1 and Table 2). The regions of INO necessary for the interaction with LUG and SEU were tested, and the data indicated that the N-terminal region of INO (INOΔC), including the zinc finger region, was sufficient to promote the interaction with the LUG and SEU proteins (Figure S3 in File S1 and Table 2). There was also evidence of some interaction of SEU with the C-terminal region of INO (INOΔN) (Figure S3 in File S1 and Table 2).
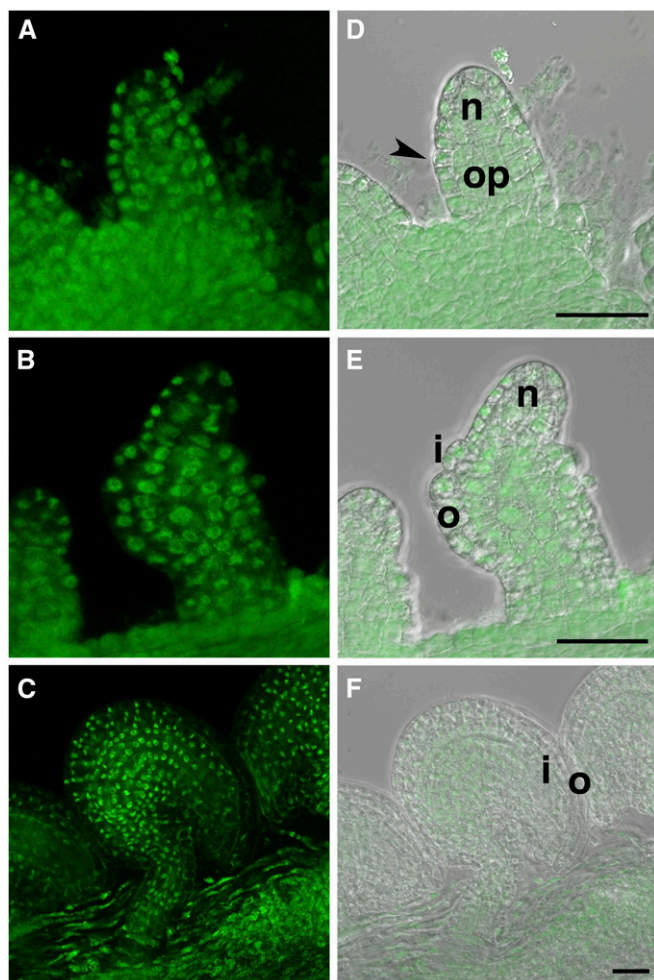
Figure 2 Accumulation of ADA2b-GFP (PRZ-GFP) fusion in ovule cells. The expression pattern of a P-PRZ1::PRZ1:GFP (P-ADA2b::ADA2b:GFP) fusion in a genomic fragment that complemented the *prz1* mutant (Sieberer *et al.* 2003; Anzola *et al.* 2010) was evaluated by laser confocal microscopy. Left panels are GFP fluorescence and the right panels are the fluorescent images overlaid on differential interference contrast images. Images are of ovules at stages: (A and D) 2-I; (B and E) 2-IV; and (C and F) 4-V. Expression is in all cells at all stages of ovule development and the fusion protein accumulates in the nucleus. Arrowhead, site of outer integument initiation; i, inner integument; n, nucellus; o, outer integument; and op, ovule primordium. Bar, 25 μm.

Roles of LUG and SEU in ovule development

Both *LUG* and *SEU* are expressed in ovules, including the cells known to express *INO* (Conner and Liu 2000; Bao *et al.* 2010). Previous reports have also shown that *lug* and *seu* mutants exhibit ovule integument defects characterized by a reduction in outer integument growth relative to the inner integument (Roe *et al.* 1997; Franks *et al.* 2002). Here, we observed similar phenotypes of an exposed portion of the inner integument at anthesis, but note that the initiation and growth of the outer integument appeared similar to wild-type at early stages of ovule development for both mutants (Figure 4).

As in the *ino-1* single mutant (Figure 4, A and B), in *lug-1 ino-1* and *seu-1 ino-1* double mutants the outer integument

was not initiated (Figure 4, G, H, K, and L). Thus, the *ino-1* phenotype in the outer integument was epistatic to both the *lug-1* and *seu-1* mutant phenotypes. The absence of the outer integument in the *ino-1 lug-1* and *ino-1 seu-1* mutants also allowed a more complete examination of the growth and development of the inner integument. *lug-1 ino-1* did not differ from the *ino-1* single mutant and the inner integument covered the nucellus at anthesis (Figure 4L). In contrast, in *seu-1 ino-1*, the extent of inner integument growth was reduced, resulting in ovules with an exposed nucellus at anthesis (Figure 4, G and H). Unlike the *ada2b-1* mutant, which showed both reduced inner integument growth and also gaps or divisions in the overall structure of the integument, the continuous structure of the inner integument was maintained in *seu-1* mutants. In contrast to the *ada2b-1 ino-1* mutant, mutant combinations with *seu-1* and *lug-1* did not alter the funiculus or affect the gynoapical outgrowth on the chalaza and maintained the distinct groove at the base of the inner integument.

Genetic interaction of lug and ada2b

While effects of the *ada2b*, *lug*, and *seu* mutants included reduced outer integument growth, the growth of this structure was always greater than observed in the strong *ino-1* allele or even the weaker *ino-4* allele (Villanueva *et al.* 1999; Figure 3 and Figure 4). This indicates that some INO function was present to promote partial growth of the outer integument in both coactivator and corepressor mutant lines. Thus, we combined the two classes of coregulator mutations, producing *lug-1 ada2b-1* double mutants, and found that this produced greater defects in outer integument growth than either single mutant, as well as other effects on ovule development (Figure 5). The outer integument of the *lug-1 ada2b-1* mutant initiated on the correct gynobasal side of the ovule and grew asymmetrically around the ovule, but the growth of the outer integument was deficient at earlier stages in ovule development than in either single mutant (Figure 5). While some ovules simply had reduced outer integuments (Figure 5A), the directional growth of the outer integument was more commonly seriously disrupted (Figure 5, B and C). For 52 of 94 ovules in 28 examined carpels, the outer integument formed a large group of cells at the chalazal region that lacked directional growth or grew in the opposite direction toward the funiculus (Figure 5, B and C). Furthermore, in some ovules, disruption of integument development was even more extreme, with multiple aberrant structures emerging from the chalaza (Figure 5C). This is a novel effect not observed in either single mutant. In all cases, there was less growth of the outer integument in the *lug-1 ada2b-1* double mutant than in either of the two single mutants. The additive and synergistic aspects of the double mutant indicate that *LUG* and *ADA2b* act independently to promote outer integument growth. As a consequence of the reduced and aberrant outer integument growth, the overall shape of the mature ovule was also compromised (Figure 5, B and C).

The addition of the *ino-1* mutation to the combination of *lug-1* and *ada2b-1* largely eliminated the production of cells
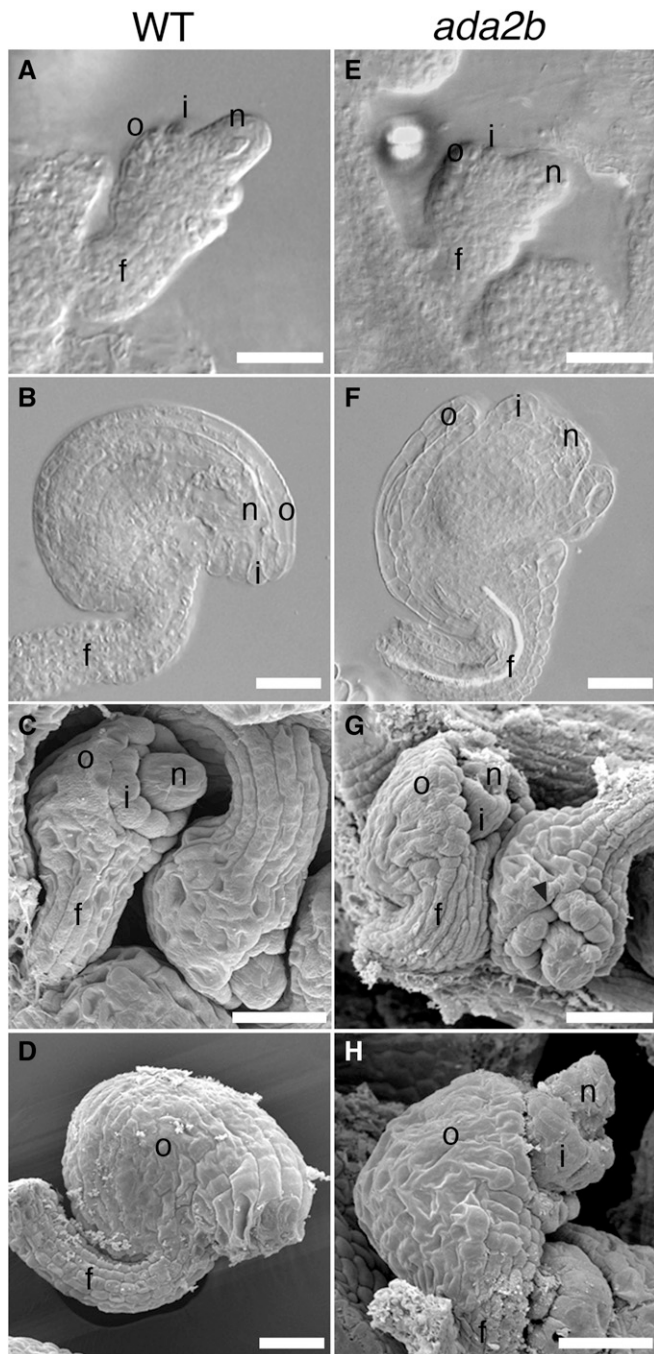
WT   *ada2b*

Figure 3 *ada2b* mutation affects ovule development. Differential interference contrast (A, B, E, and F) and scanning electron (C, D, G, and H) micrographs of wild-type (A–D) and *ada2b* (E–H) ovules. At stage 2-IV, ovules of *ada2b* (E) are similar to those of wild-type at this same stage (A). At stage 3-III, discontinuous growth of the inner integument results in fissures in this structure (arrowhead) in *ada2b* (G), while this structure is continuous in wild-type at this same stage (C). At the completion of prefertilization ovule development at stage 3-V, the integuments of wild-type ovules have covered the inner integument (B and D), while at this same stage the inner integument and nucellus are both exposed in *ada2b* due to decreased extension of the outer integument (F and H). f, funiculus; i, inner integument; n, nucellus; and o, outer integument. Bar, 25 μm.

in the region normally occupied by the outer integument (Figure 5, D–G). The resulting ovules often resemble *ino-1 ada2b-1* ovules (compare Figure 4, C and D with Figure 5, D–F). Some ovules were more aberrant, showing multiple nucelli on structures that resembled fused adjacent ovules (Figure 5G), possibly indicating defects in ovule spacing in the gynoecium.

Pleiotropic mutant phenotypes of *lug-1* in leaf and floral specification and morphology, and *ada2b-1* in cell elongation/proliferation and leaf/flower morphology, have been previously reported (Liu and Meyerowitz 1995; Liu *et al.* 2000; Sieberer *et al.* 2003; Vlachonasios *et al.* 2003; Hark *et al.* 2009). These phenotypic effects were also observed in the *lug-1 ada2b-1* double mutant, including the carpelloid sepals characteristic of the *lug-1* mutant (Liu and Meyerowitz 1995) and the reduced stature and altered leaf morphology characteristic of the *ada2b-1* mutant. However, in addition to the novel ovule phenotype, this double mutant also exhibited novel defects in the structure of the gynoecium. The *lug-1* mutant is characterized by unfused carpels with a horn-like protrusion at the tip of the carpel valve (Liu and Meyerowitz 1995) (Figure 5J), and the *ada2b-1* mutant did not show any significant carpel alterations (Figure 5I). In contrast, the *lug-1 ada2b-1* double mutant frequently developed a gynoecium with decreased ovary tissue, often forming only a single ovary (Figure 5K). This phenotype was variable in the extent of fusion of the gynoecium along the medial domain, the presence of horn-like protrusions from the carpel valve regions, and also the number of carpels.

Discussion

Mutations in *ADA2b*, *SEU*, and *LUG* all produce similar decreases in growth of the outer integument, where extension is variable but always less than that of wild-type. These observations, combined with the observation of expression of all three genes in the outer integument, demonstrate roles for the protein products of these genes in outer integument growth and extension. As a known coactivator (Vlachonasios *et al.* 2003) and two known interacting corepressors (Franks *et al.* 2002; Navarro *et al.* 2004; Sridhar *et al.* 2004), these proteins would perform their functions through regulation of transcription, but would require a partner protein with DNA-binding activity to target specific genes. The observation that these coregulators can interact with INO suggests INO as such a partner for the regulation of outer integument growth. Notably, INO is a master regulator of outer integument formation, being required for both the initiation and subsequent growth of this structure (Villanueva *et al.* 1999). The observed interaction of INO with SEU and LUG is further supported by prior observation of interaction of these corepressors with INO orthologs, other YABBY proteins in other species (Navarro *et al.* 2004), and with other YABBY proteins in *Arabidopsis* (Stahle *et al.* 2009). Our observation that ADA2b (PRZ1) can interact with a range of other YABBY proteins further supports the interaction. Indeed, evidence suggests that vegetative YABBY proteins
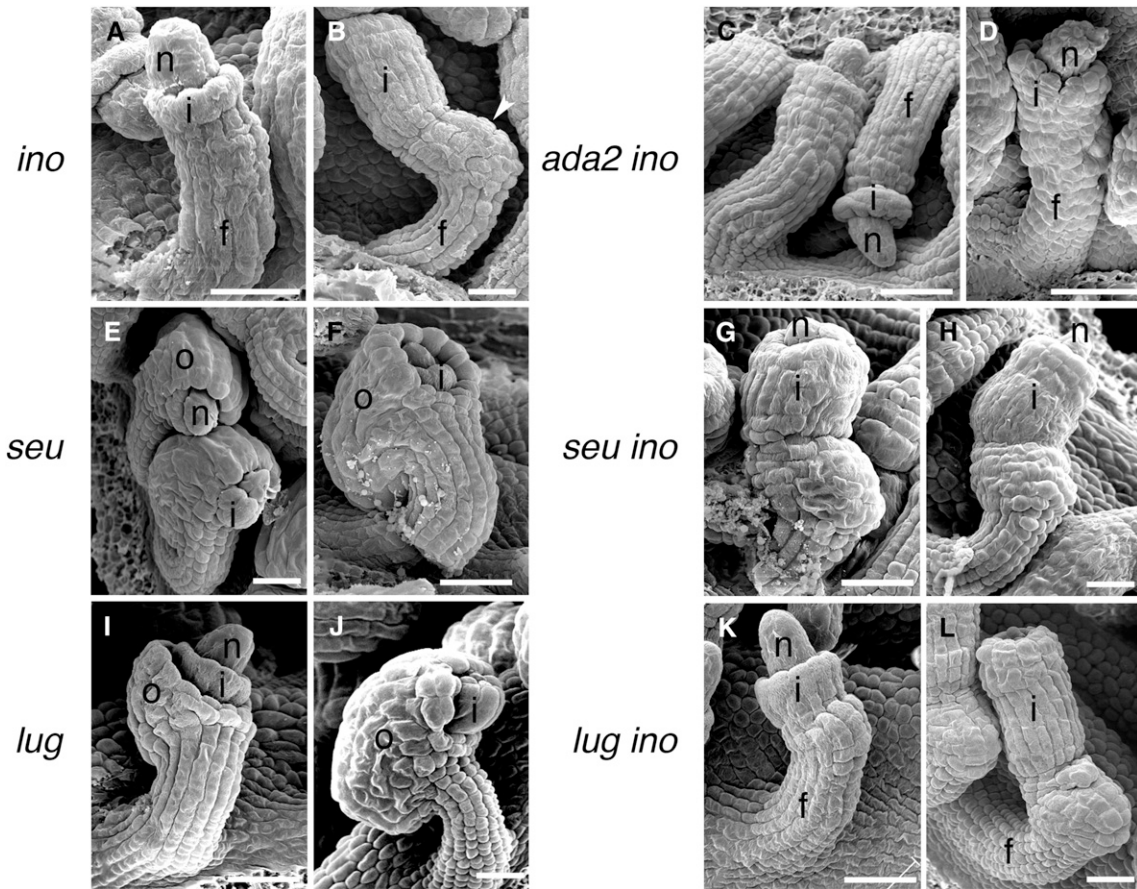
Figure 4 Interactions of *ada2b*, *lug*, and *seu* mutations with *ino* in ovule development. Scanning electron micrographs of ovules of the indicated single and double mutants are shown. As previously reported (Villanueva *et al.* 1999), *ino-1* ovules initiate only an inner integument (A) and lack outer integuments at maturity (B). The inner integument and a thickening of the chalazal region (arrowhead) are exposed by the absence of the outer integument. In the combination of *ada2b* with *ino* (C and D), the chalazal thickening is absent, the boundary at the insertion of the inner integument is often indistinct, and fissures are visible in the inner integument (D). Both *seu* and *lug* mutants initiate both integuments (E and I), but the outer integuments of both mutants exhibit reduced growth and fail to cover the inner integument at maturity (F and J). The inner integument of *seu* mutants also shows reduced growth and fails to cover the nucellus at maturity (F), and this is more clearly visible when the outer integument is eliminated in the *seu ino-1* double mutant (G and H). The inner integument appears unaffected in *lug* and in the *lug ino-1* double mutant (K and L). f, funiculus; i, inner integument (primordium); n, nucellus; and o, outer integument. Bar, 25 μm.

have both negative and positive effects on transcription (Bonaccorso *et al.* 2012), and our results would provide a mechanistic explanation for this.

Our observation that a severe *ino* loss-of-function mutation was epistatic to *seu*, *lug*, and *ada2b* mutations in the outer integument is what is expected if INO is a tether enabling the gene-specific function of these coregulators in the outer integument. The effects of mutations in these coregulators on other plant processes outside of the outer integument would involve DNA-binding proteins other than INO and so are not altered by *ino* mutations. The repressive effects of the corepressors and the activating effect of the ADA2b coactivator would be expected to be directed at different sets of genes in outer integument growth. Eliminating INO as a vector of both coactivators and corepressors would be expected to have more extreme effects than mutations in either single class of coregulator. This is indeed what we observed, with complete loss of the outer integument in the strong *ino* mutants,

and only partial loss of outer integument growth observed for either loss of coactivator or corepressor function. This model is further supported by the observation that effects of combining corepressor and coactivator mutations (in the *ada2b lug* mutant) are both additive and synergistic. Mutants with defects in genes in both classes would be expected to have a more severe effect on growth of the outer integument than either single coregulator mutation, and this is what was observed. However, some outer integument growth persisted in the *ada2b lug* double mutant, but this was eliminated by the addition of an *ino* mutation. This indicated participation of INO in the residual outer integument growth. We note that a paralog of *LUG*, *LUH*, has been shown to have activity that is partially redundant with the activity of *LUG* (Sitaraman *et al.* 2008), and this could account for the residual outer integument growth activated by *INO* in the *ada2b lug* double mutant.

This model for INO as a tether binding the coregulators to DNA relies on INO being a DNA-binding transcription factor or

Table 2 Yeast two-hybrid analysis of INO interactions with INO, LUG, and SEU

BD	AD			
	Empty	INO	LUG	SEU
Empty	—	—	—	—
INO	—	+++	+++	+++
INOΔC	—	+++	+++	+++
INOΔN	—	—	—	+

AD, activation domain; BD, DNA-binding domain.

a bridge to such a factor. INO is nuclear-localized (Meister *et al.* 2002), as would be expected for a transcription factor. Initial attempts to directly identify sequences specifically bound by a YABBY protein (FIL) found only weak, nonspecific DNA-binding activity (Kanaya *et al.* 2002). However, more recently, Franco-Zorrilla *et al.* (2014) used DNA microarrays to detect binding of FIL and YAB3 to specific DNA sequences. Also, using ChIP, Shamimuzzaman and Vodkin (2013) were able to detect binding of soybean FIL/YAB3 orthologs to specific sites in the genome and were able to find evidence for a consensus binding motif that was different from the motif identified in the microarray study. The latter study supports YABBY proteins as DNA-binding proteins *in vivo*, where there might be additional factors (including other necessary proteins) facilitating DNA binding that are absent in *in vitro* and heterologous assays. If INO is a DNA-binding protein then our observation of an ability of INO to interact with itself via the YABBY domains could indicate that it acts as a homodimer. Prior work has shown both homo- and heterodimerization between YABBY proteins (Kanaya *et al.* 2001; Stahle *et al.* 2009). However, because *INO* is the only YABBY gene that has been shown to be expressed in ovules (Bowman and Smyth 1999; Villanueva *et al.* 1999), only the homodimer is a possibility for INO protein *in vivo*.

Evaluation of the regions of INO binding ADA2 and LUG/SEU indicates that the two classes of proteins have differential affinity for different INO regions. While some binding was detected for each class to both the N- and C-terminal regions of INO, binding of the coactivator was strongest to the C-terminal region of INO, while binding of corepressors was preferential to the N-terminal region. These results indicate that, like some other transcription factors (Kurokawa *et al.* 1995), INO could potentially simultaneously interact with both corepressors and coactivators.

Both ADA2b and the paralogous ADA2a were found to interact with INO. The absence of phenotypic effects of the *ada2a* mutation, even in combination with an *ada2b* background, led us to not study *ada2a* mutants further. Evidence for ADA2b as a coactivator that facilitates recruitment of the HAT GCN5 to DNA-binding factors is strong (Vlachonasios *et al.* 2003; Hark *et al.* 2009; Kornet and Scheres 2009; Anzola *et al.* 2010). This type of mechanism has been demonstrated for the activation of cold response genes via transcription factor CBF1 recruitment of ADA2b and GCN5 (Stockinger *et al.* 2001; Mao *et al.* 2006). The HAT activity

of GCN5 is recruited by other transcription factors to affect a wide variety of additional processes in plants, including maintenance of the stem cell niche (Kornet and Scheres 2009), floral meristem activity (Bertrand *et al.* 2003), light responses (Benhamed *et al.* 2006), and auxin responses (Anzola *et al.* 2010; Weiste and Droge-Laser 2014). To these, we would add a likely involvement in ovule development through interaction with INO, and possibly participation in polarity determination via interaction with other members of the YABBY gene family. Notably, tubular or filamentous growths were observed in the inflorescence of the *gcn5* mutant, and this resembles effects of *fil* mutants (Cohen *et al.* 2009). This is consistent with *GCN5/ADA2b* also functioning in the lateral organ YABBY pathway.

Several lines of evidence support the function of SEU and LUG acting as a transcriptional corepressor complex (Sridhar *et al.* 2004; Stahle *et al.* 2009; Grigorova *et al.* 2011). While we studied these genes individually, mutant combinations of *lug* and *seu* did not lend themselves to the study of mutant ovules because they cause such severe effects on plant and flower growth that formation of ovule-like structures is rare (Franks *et al.* 2002). This is likely due to participation in multiple associations with a variety of transcription factors during gynoecium development (Pfluger and Zambryski 2004; Sridhar *et al.* 2006), likely including other YABBY proteins (Stahle *et al.* 2009). The effects of *lug* and *seu* mutations are likely not indications of the complete loss-of-function of the corepressor activity because both have at least partially redundant paralogous proteins: LUH (Sridhar *et al.* 2004) and SLK1/2/3 (Bao *et al.* 2010). Thus, the mutations examined in this work may not show the full extent of participation of these classes of corepressors in ovule development. Recently, evidence that LUH might also participate in transcriptional activation as well as repression has been reported (Lee *et al.* 2015), and LUG/LUH associations could therefore also partly account for gene activation by YABBY proteins.

The challenge of determining the actual biochemical role of proteins genetically identified as being important in development is daunting. It is facilitated by the incremental assembly of multiple pieces of independently acquired evidence, as undertaken in the current work. The importance of YABBY proteins in the development and polarity of lateral organs and ovule integuments is well established (Bowman and Smyth 1999; Villanueva *et al.* 1999; Sarojam *et al.* 2010). The specific patterns of expression of these genes are consistent with their direct participation in the development of these structures (Bowman and Smyth 1999; Villanueva *et al.* 1999). Their nuclear localization and similarities to known DNA-binding proteins suggested that they might be DNA-binding transcription factors. ChIP and DNA-binding assays now provide further support for this (Shamimuzzaman and Vodkin 2013; Franco-Zorrilla *et al.* 2014). Prior results indicating interactions with SEU and LUG (and both paralogous proteins and orthologs in other species) provided a first indication of a possible mechanism of action, but did not explain the apparent positive and negative action on gene expression.
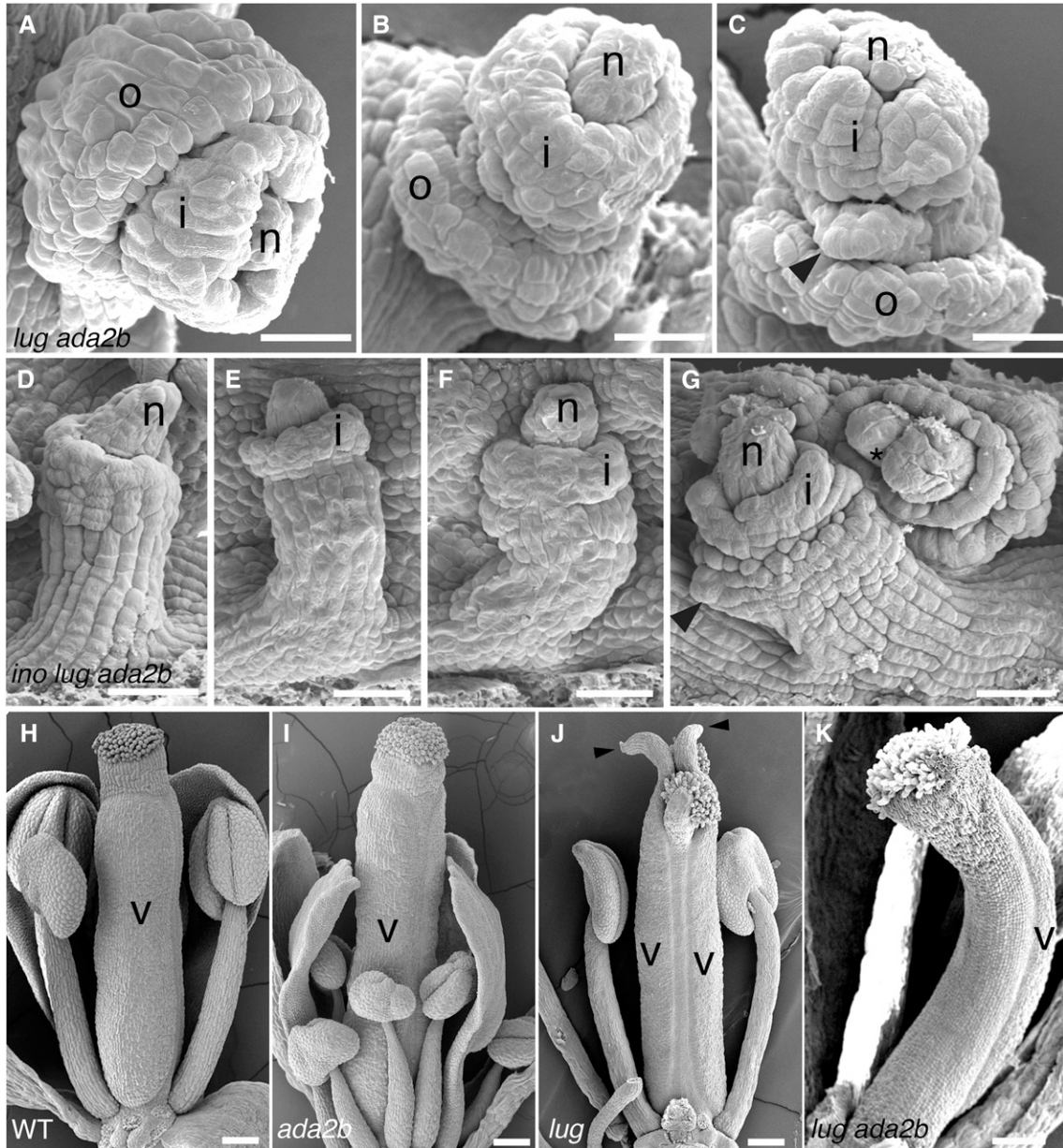
Figure 5 Synergistic interactions of *ada2b* and *lug* mutations on ovule and carpel development. (A–C) ovules of *lug ada2b* double mutants at stage 12. While occasional ovules have a relatively normal but reduced outer integument (A), the majority of ovules show a synergistic effect on the outer integument, where it forms an amorphous collar of tissue (B and C). The inner integument may also be affected (C). Ovules of the *lug ada2b ino* triple mutant at stage 12 (D–G) most closely resemble those of the *ino ada2b* double mutant, with an absent outer integument and often indistinct base of the inner integument (D). Growth to stage 13 shows aberrant expansion of the funiculus, which can protrude up around the inner integument (G) (arrowhead). Some very aberrant ovules can have two nucelli (G) (*), indicating probable fusion of two ovule primordia. Carpels of *ada2b* mutants (I) resemble those of wild-type (H), but *lug* mutants have aberrant carpels (J) with horn-like protrusions (arrowheads) interrupting the stigma. Such horns are absent in *lug ada2b* double mutants (K) and carpel tissue is reduced such that often only a single valve is present in the gynoecium. i, inner integument (primordium); n, nucellus; o, outer integument; and v, valve. Bar, 20 μm in (A–C), 25 μm in (D–G), and 100 μm in (H–K).

Our results showing interaction with both coactivator and corepressor complexes now provide a possible mechanism for these conflicting activities. Additional interactions of the YABBY proteins with other proteins would be necessary to provide the discrimination required to differentially associate the activating and repressive complexes with their specific downstream target genes. Such factors might be expected to recognize both a component of the repressor or activator complex as well as target genes using their regulatory sequences. At least one possible candidate for this may have already been described. The NOZZLE/SPOROCYTELESS (NZZ/SPL) protein has been shown to associate with INO (Sieber *et al.* 2004) and has more recently been shown to repress expression of specific genes through association with the TOPLESS (TPL) corepressor (Wei *et al.* 2015). An association with INO could form a multifunctional repressive

complex combining the LUG/SEU and TPL activities to target specific genes during integument development. Alternative associations could specifically target INO bound to ADA2b to a different set of genes for activation.

Further insight could be provided by the identification of direct targets of *INO* regulation and determination of the subset of genes regulated by *INO* that are also regulated by *ADA2b* or by *LUG* and *SEU*. Interestingly, the *DAMAGED DNA-BINDING PROTEIN 1B* (*DDB1B*) gene [encoding part of the CULLIN4 (CUL4) E3 ligase that acts in photomorphogenesis (Schroeder *et al.* 2002; Bernhardt *et al.* 2006)] is upregulated 19-fold in flowers of *lug* mutants (Gonzalez *et al.* 2007), and is also upregulated 20-fold in *ino* mutant pistils (Skinner and Gasser 2009). Thus, this gene could be a target of an INO/LUG/SEU complex. The *ETTIN* (*ETT*) gene is upregulated twofold in both *lug* flowers and *ino* pistils (Gonzalez *et al.* 2007; Skinner and Gasser 2009). *ETT* expression must be repressed in the outer integument for normal development of this structure (Kelley *et al.* 2012) and so *ETT* is another potential target for a repressive INO/LUG/SEU complex. Additional potential targets for repression and for activation by INO remain to be discovered.

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