

ETTIN (ARF3) physically interacts with KANADI proteins to form a functional complex essential for integument development and polarity determination in *Arabidopsis*

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SUMMARY

KANADI (KAN) transcription factors promote abaxial cell fate throughout plant development and are required for organ formation during embryo, leaf, carpel and ovule development. *ABERRANT TESTA SHAPE* (*ATS*, or *KAN4*) is necessary during ovule development to maintain the boundary between the two ovule integuments and to promote inner integument growth. Yeast two-hybrid assays identified ETTIN (*ETT*, or AUXIN RESPONSE FACTOR 3) as a transcription factor that could physically interact with *ATS*. *ATS* and *ETT* were shown to physically interact in vivo in transiently transformed tobacco epidermal cells using bimolecular fluorescence complementation. *ATS* and *ETT* were found to share an overlapping expression pattern during *Arabidopsis* ovule development and loss of either gene resulted in congenital fusion of the integuments and altered seed morphology. We hypothesize that in wild-type ovules a physical interaction between *ATS* and *ETT* allows these proteins to act in concert to define the boundary between integument primordia. We further show protein-protein interaction in yeast between *ETT* and *KAN1*, a paralog of *ATS*. Thus, a direct physical association between *ETT* and *KAN* proteins underpins their previously described common role in polarity establishment and organogenesis. We propose that *ETT-KAN* protein complex(es) constitute part of an auxin-dependent regulatory module that plays a conserved role in a variety of developmental contexts.

KEY WORDS: Ovule, Seed, Organ fusion, Transcription factor, Abaxial, *Arabidopsis*

INTRODUCTION

Ovules are seed precursors and typically have two sheathing structures, termed the inner and outer integuments, that form the seed coat following fertilization. Numerous genes involved in integument patterning and growth have been identified from forward genetic screens (Skinner et al., 2004; Colombo et al., 2008). One such gene, *ABERRANT TESTA SHAPE* (*ATS*) encodes a KANADI (KAN) transcription factor (TF). *KAN* genes provide patterning and growth cues during embryogenesis (Izhaki and Bowman, 2007), lateral root formation (Hawker and Bowman, 2004), adaxial-abaxial leaf polarity establishment (Eshed et al., 2001; Kerstetter et al., 2001; Eshed et al., 2004) and integument formation (Eshed et al., 2001; McAbee et al., 2006; Kelley et al., 2009). Loss of *ATS* function leads to congenital fusion of the inner and outer integuments and abnormal seed formation (Leon-Kloosterziel et al., 1994; McAbee et al., 2006). *ATS* plays dual roles during ovule development, providing boundary maintenance and promoting laminar growth of the inner integument (McAbee et al., 2006). Similarly, loss of *KAN1* and *KAN2* disrupts laminar growth of the outer integument (Eshed et al., 2001; McAbee et al., 2006). Thus, these genes play similar roles but *ATS* acts in the inner

integument whereas *KAN1* and *KAN2* act redundantly in the outer integument (Leon-Kloosterziel et al., 1994; Eshed et al., 2001; McAbee et al., 2006).

In vegetative organs there is substantial functional overlap between KAN family members [*KAN1*, *KAN2*, *KAN3* and *ATS* (*KAN4*)] (Eshed et al., 2001; Izhaki and Bowman, 2007). During embryo development, *KAN1*, *KAN2* and *ATS* are required to restrict polar *PIN-FORMED 1* (*PIN1*) expression from the hypocotyl and subsequently maintain unidirectional auxin flows towards the cotyledon primordia (Izhaki and Bowman, 2007). Additionally, genetic data suggest that this regulation of auxin signaling might occur by the cooperative action of KAN and AUXIN RESPONSE FACTOR (ARF) proteins (Pekker et al., 2005). Specifically, the suppression of effects of ectopic *KAN1* activity by loss of *ETTIN* (*ETT*, also known as *ARF3*) function and the resemblance of *ett arf4* double mutants to *kan1 kan2* double mutants suggests that these different TFs have similar functions during organogenesis (Pekker et al., 2005). Because *kan* mutants and *ett arf4* mutants display both unique and shared phenotypes, KAN and ETT/ARF4 appear to act both in concert and in independent roles.

Although many of the genes involved in plant development are known to encode TFs, our current understanding of the transcriptional complexes that are active during organ formation is incomplete. This is especially true for organs that are small and/or difficult to mechanically isolate from other tissues, such as ovules. Definition of protein-protein interactions during ovule development is required to further our understanding of how TFs act at the molecular level to integrate hormone signaling and organogenesis. The substantial overlap in genes active during ovule and leaf development (Kelley and Gasser, 2009) suggests that transcription partners might be conserved, providing genetic modules that act repetitively throughout plant development. Here we show that ETT and *ATS* form a functional complex active in ovule development

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and provide evidence that a similar complex between ETT and other KAN proteins underlies the cooperative activity of these proteins in leaf development.

MATERIALS AND METHODS

Plasmids and cDNA clones

ATS cDNA was amplified from P_{OP}:KAN4 (Hawker and Bowman, 2004) by PCR, digested with *Nde*I/*Nco*I and inserted into pGBKT7 (Clontech), creating pDK8 [GAL4 DNA-binding domain (BD)-*ATS*]. *ATS* cDNA without the stop codon was amplified from P_{OP}:KAN4 and inserted into pENTR/D-Topo (Invitrogen), creating pAA34. *ATS* cDNA was amplified from P_{OP}:KAN4 and inserted into pENTR4 (Invitrogen), creating pDK23. pDK23 was Gateway cloned into pDEST-GADT7 (Rossignol et al., 2007) to create pDK131 [GAL4 activation domain (AD)-*ATS*]. *KAN2* cDNA and *ETT* cDNA were amplified from wild-type Columbia (Col) leaf cDNA and inserted into pENTR/D-Topo, creating pAA29 and pDK132, respectively. *ETT* cDNA without the stop codon was amplified from leaf cDNA and cloned into pENTR/D-Topo to create pDK74. pAA29 was Gateway cloned into pDEST-GBKT7 (Rossignol et al., 2007) creating pAA36 (BD-KAN2). pDK132 was Gateway cloned into pDEST-GADT7 creating plasmid pDK136 (AD-*ETT*). *ETT* cDNA was cloned as an *Nde*I/*Xho*I fragment from pDK136 into pGBKT7 using *Nde*I/*Sal*I to create pDK137 (BD-*ETT*). *ARF4* cDNA was amplified from Col leaf cDNA and cloned into pENTR/D-Topo, creating pAA30. pAA30 was Gateway cloned into pDEST-GADT7 to create pAA42 (AD-*ARF4*). *KAN1* cDNA was amplified and inserted as a *Bam*HI/*Pst*I fragment into pLITMUS28 to create pLMK37 and transferred as a *Bam*HI/*Pst*I fragment into: (1) pGAD424, creating pLMK44 (AD-KAN1); and (2) pAS2, creating pLMK46 (BD-KAN1).

A 700 bp subclone of *ETT* (pDK73) cDNA was generated from pAS13 as described (Sessions et al., 1997). *ATS*-eGFP (pDK77) was created by Gateway cloning pAA34 into pDH51-GW-eGFP (Zhong et al., 2008). *ETT*-eGFP (pDK80) was created by Gateway cloning pDK74 into pDH51-GW-eGFP. Subclones for *ATS*-YFPc (pCG51) and *ETT*-YFPn (pCG54) were created by amplifying *ATS* or *ETT* cDNA without stop codons and inserting the resulting fragments into pJET1.2 (Fermentas) to form pCG45 and pCG46, respectively. The cDNAs were inserted into 2X35S-SPYCE or 2X35S-SPYNE vectors (Walter et al., 2004), respectively, as *Xho*I/*Xma*I fragments into these same sites forming pCG47 and pCG50. The resulting expression cassettes were transferred into pMLBART (Gleave, 1992) as *Not*I fragments, producing pCG51 and pCG54. Gateway reactions were performed using LR Clonase II (Invitrogen). All plasmids were sequence verified. Primers are listed in supplementary material Table S1.

Yeast two-hybrid assays

Yeast transformations, matings and cDNA library preparation were performed according to the Matchmaker Yeast Transformation System 2 manual (Clontech). Tissue for a cDNA library was prepared from *Arabidopsis* pistils from stage 9-12 flowers (Smyth et al., 1990). Total RNA was extracted from the harvested tissue and used for cDNA library construction using the BD Matchmaker Library Construction and Screening Kit (Clontech); the cDNA library was transformed into AH109. For the initial screen, pDK8 (BD-*ATS*) was transformed into Y187 (Clontech) and then mated with the pistil library. Mating mixtures were screened on yeast media lacking leucine (LEU), tryptophan (TRP), adenine (ADE) and histidine (HIS). Inserts were amplified from yeast cultures using the Illustra TempliPhi Amplification Kit (GE Healthcare) and directly sequenced. Plasmids were extracted from yeast using the Zymo II Yeast Plasmid Miniprep Kit (Zymo). Pairwise interaction tests were performed by directly transforming plasmids into AH109 and replica plating the transformants on yeast media lacking (1) LEU and TRP and (2) LEU, TRP, ADE and HIS. Colonies were restreaked on similar media at least twice for verification.

Plant material

Arabidopsis plants were grown under long-day conditions as previously described (McAbee et al., 2006). The alleles used in this study in a Col-0 background were *ats-2* (McAbee et al., 2006) and *arf3-2* (Okushima et al., 2005). Other alleles examined are in a *Ler* (*ats-1*, *ett-3*) or a *Ws-2* (*arf4-1*, *ett-1*, *ett-2*) background (Sessions et al., 1997; Pekker et al., 2005).

Transient expression studies and bimolecular fluorescence complementation (BiFC) assays

Transient expression of protein fusions *ATS*-eGFP (pDK77) and *ETT*-eGFP (pDK80) in onion epidermal cells was performed as described (Skinner et al., 2001). A 35S:β-glucuronidase control plasmid (pHK17) (Norris et al., 1993) was co-bombarded as a positive control for cell transformation. After imaging for fluorescence, staining for GUS activity was performed as described (Skinner et al., 2001) and the positively transformed cells were counted.

BiFC assays were performed in triplicate as described (Szemenyei et al., 2008). The abaxial surfaces of infiltrated tobacco leaves were imaged 2-5 days after inoculation.

In situ hybridization

Tissue preparation, in situ hybridization and *ATS* probe synthesis were performed as described (Kelley et al., 2009). The *ETT* probe was generated by digesting pDK73 with *Bam*HI and transcribing with T7 RNA polymerase (Promega) and DIG labeling mix (Roche).

Microscopy

Scanning electron microscopy and ovule clearings were performed as described (Kelley et al., 2009). Seeds were imaged using a SPOT RT Slider camera system (SPOT Imaging Solutions) on a Leica MZ FLIII dissecting scope. Bright-field and fluorescence images of onion cells were acquired using a Kodak MDS290 camera on a Zeiss Axioplan microscope with an Endow GFP bandpass emission 41017 filter set (Chroma Technology). Bright-field and fluorescence images of tobacco cells were acquired using a SPOT RT Slider camera system on a Leica DM5000B microscope with a GFP filter set. Images were edited in Adobe Photoshop CS2.

RESULTS AND DISCUSSION

Yeast two-hybrid screen with *ATS* identifies *ETT*N

To identify protein complex(es) in which *ATS* might participate during ovule development we performed a yeast two-hybrid screen using full-length *ATS* as bait and a pistil cDNA library as prey. We obtained a number of positive clones, including a clone encoding the DNA-binding domain of *ETT* (amino acids 12-34) (Ulmasov et al., 1999). We subsequently produced fusions of full-length *ETT* to both the GAL4 DNA-binding domain (BD) and activation domain (AD) and showed that full-length *ATS* and *ETT* interact in yeast irrespective of the GAL4 fusion protein orientation (supplementary material Fig. S1). We examined interactions of *ATS* paralogs *KAN1* and *KAN2* with *ETT* and also of these proteins with the *ETT* paralog *ARF4* (supplementary material Fig. S1). These studies revealed an interaction between *KAN1* and *ETT*, but BD-KAN2 alone showed auto-activation in this yeast system, masking any possible interactions (supplementary material Fig. S1). None of the other tested fusion proteins showed auto-activation of the reporter genes (supplementary material Fig. S1). The yeast assays did not provide evidence for interaction between *ARF4* and *KAN* proteins. Thus, at least two different *KAN* proteins can bind *ETT* in yeast. Differences in the protein-protein interactions of *ETT* and *ARF4* might result from structural differences between these proteins, as *ETT* lacks the conserved domains III and IV that are present in *ARF4*.

ATS and *ETT* can physically interact in the plant cell nucleus

In onion epidermal cells, transient expression of translational fusions of *ATS* or *ETT* to eGFP led to fluorescence that was observed only in nuclei (Fig. 1B,D), confirming nuclear localization of these two TFs. Transient co-expression of a translational fusion of *ATS* to the C-terminal portion of yellow fluorescent protein (YFP) (*ATS*-YFPc) and of *ETT* to the N-terminal portion of YFP (*ETT*-YFPn) showed BiFC (Kerppola,

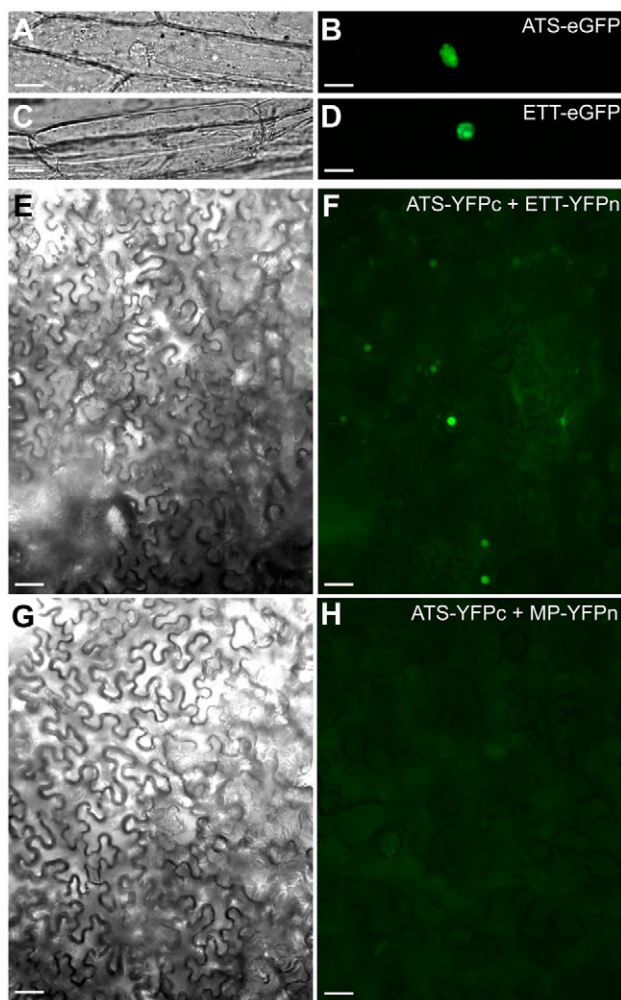


Fig. 1. ATS and ETT are nuclear localized and physically interact in vivo in a BiFC assay. (A–D) ATS-eGFP (A,B) and ETT-eGFP (C,D) are nuclear localized. (E,F) Fluorescence indicates direct interaction in vivo between ATS and ETT that is restricted to nuclei of transformed tobacco cells. (G,H) Co-transformation of ATS and a different ARF protein, MP, fails to show fluorescence complementation. Scale bars: 20 μ m in A–D; 50 μ m in E–H.

2006) in the nuclei of tobacco epidermal cells (Fig. 1E,F). As a control, we performed similar tests for protein interaction between ATS and another ARF family member, MONOPTEROS (MP), which has been shown to be nuclear localized and to interact with a TOPLESS fusion protein in the same BiFC assay (Szemenyei et al., 2008). ATS-YFPc and MP-YFPn (Fig. 1G,H) did not show fluorescence complementation in tobacco cells, confirming that the observed BiFC between ATS and ETT was specific. These results demonstrate that ATS and ETT can directly interact in planta.

ATS and ETT are co-expressed in the inner integument during ovule development

We examined *ATS* and *ETT* expression during ovule development by in situ hybridization to establish the biological relevance of the ATS-ETT physical interaction. *ATS* expression is restricted to the abaxial region of the inner integument in both young and mature ovules (Fig. 2A,B) (McAbee et al., 2006). *ETT* was expressed in the same pattern as *ATS* during ovule development, with *ETT*

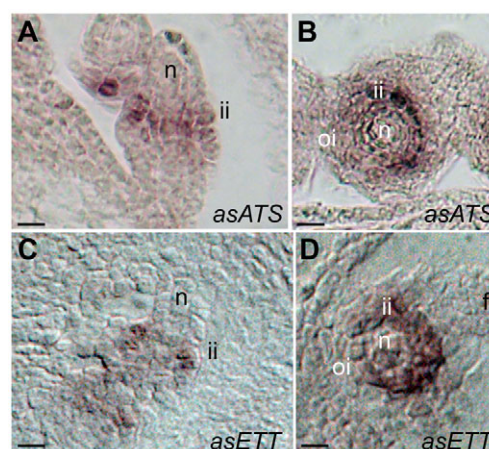


Fig. 2. ATS and ETT display overlapping mRNA accumulation patterns during ovule development. (A–D) *ATS* (A,B) and *ETT* (C,D) in situ hybridizations on young (A,C) and mature (B,D) wild-type *Arabidopsis* ovules show an inner integument-specific signal. f, funiculus; n, nucellus; ii, inner integument; oi, outer integument. Scale bars: 5 μ m in A,C; 10 μ m in B,D.

transcript first appearing during inner integument initiation and persisting throughout ovule development (Fig. 2C,D). This finding is consistent with prior transcriptional profiling (Skinner and Gasser, 2009) and hybridization studies (Ng et al., 2009). The coincident expression patterns of *ATS* and *ETT* in ovules shows that the interaction between these proteins as observed in yeast and in transgenic plant cells could occur during the normal expression of these genes.

Ovule and seed phenotypes of ett mutants resemble those of ats

During wild-type ovule development the outer integument forms a hood-like structure covering the inner integument and the nucellus (Fig. 3A,E). In *ats* mutant ovules the inner and outer integument cell layers grow as a unit, producing a single fused structure (Leon-Kloosterziel et al., 1994; McAbee et al., 2006) (compare Fig. 3B,F with 3A,E). As a result of this fusion, *ats* seeds are abnormally rounded and variable in size (Fig. 3J) (McAbee et al., 2006) compared with the uniformly elongate mature wild-type *Arabidopsis* seeds (Fig. 3I). Examination of *ett* mutant ovules (Fig. 3C,G) and seeds (Fig. 3K and supplementary material Fig. S2) revealed that they phenotypically resemble *ats* ovules and seeds (compare Fig. 3C with 3B, 3G with 3F and 3K with 3J; seeds in supplementary material Fig. S2). *ats ett* double-mutant ovules and seeds showed no phenotypic differences to either single mutant (Fig. 3D,H,L). Thus, loss of either ATS or ETT is sufficient to disrupt a common regulatory pathway that is mediated by both TFs. The severely compromised gynoecia of *arf4-1 ett-1* double mutants preclude examination of the ovules of this mutant combination. However, the wild-type morphology of *arf4 ett/+* ovules and seeds (supplementary material Fig. S2) indicates that *ARF4* is not required for integument development.

A model for ATS-ETT action during ovule development

KAN, ETT and ARF4 have been proposed to act as transcriptional repressors (Tiwari et al., 2003; Wu et al., 2008; Causier et al., 2011). We therefore speculate that an ATS-ETT TF complex acts

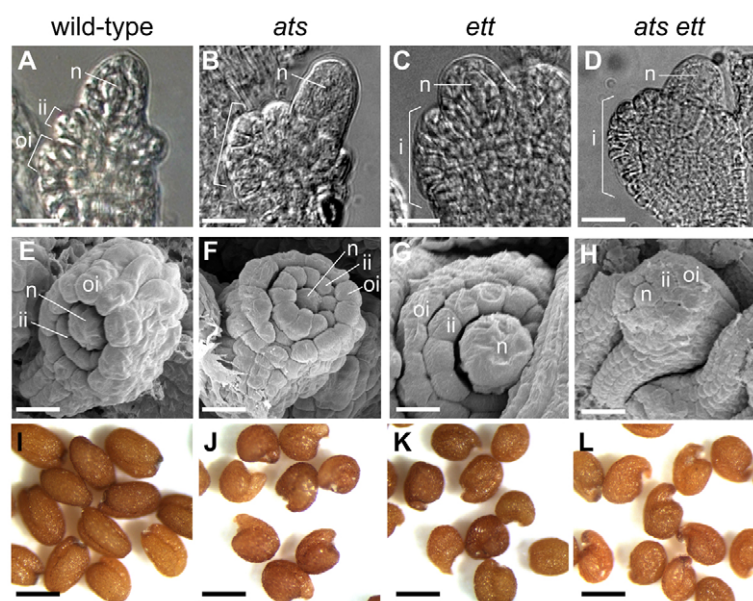


Fig. 3. Loss of *ATS* or *ETT* results in integument fusion and altered seed morphology. (A-I) Wild-type (A,E,I), *ats* (B,F,J), *ett* (C,G,K) and *ats ett* (D,H,L) ovules (A-H) and seeds (I-L). Wild-type ovules initiate two distinct integument primordia (A). Integument formation in *ats*, *ett* and *ats ett* leads to a single integument primordium (B-D). The outer integument grows over the inner integument in wild-type ovules (E), whereas the fused integuments in *ats*, *ett* and *ats ett* mutants grow in a single plane (F-H). Abnormal integument formation in *ats*, *ett* and *ats ett* mutants gives rise to aberrant seed morphology (compare J-L with I). n, nucellus; ii, inner integument; oi, outer integument; i, integument. Scale bars: 5 μm in A-D; 10 μm in E-H; 200 μm in I-L.

to repress the expression of specific genes in the abaxial domains of developing inner integuments. Furthermore, the action of such an ATS-ETT module might be directly linked to auxin signaling. PIN1-dependent auxin maxima have been shown to occur in ovule and integument primordia (Benkova et al., 2003). KAN proteins play a role in restricting PIN activity and thus auxin flow during embryogenesis (Pekker et al., 2005; Izhaki and Bowman, 2007; Ilegems et al., 2010). PIN proteins regulate auxin flow, and auxin has been shown to control PIN gene expression as well as PIN cellular polarity via the TIR1-Auxin/IAA-ARF pathway (Schrader et al., 2003; Vieten et al., 2005; Sauer et al., 2006).

Based on these observations, we propose a model for ATS-ETT action during ovule development (Fig. 4). Initially, an auxin maximum occurs at the apex of the nucellus (Fig. 4A). Following

integument initiation, the ATS-ETT complex accumulates in the abaxial layer of the inner integument and refines auxin action in the chalaza through negative regulation of PIN1 and hence auxin transport (Fig. 4B). Mutation in either protein eliminates this resolution, leading to the formation of a single broad integument (Fig. 4C). Auxin is also known to positively regulate *ETT* activity (Tiwari et al., 2003). Positive feedback from auxin levels on ETT combine with ATS-ETT suppression of PIN (and hence auxin levels) to establish and homeostatically maintain the appropriate level of ETT, PIN1 and auxin activity necessary for lateral organ outgrowth (Fig. 4D).

Our model for inner integument outgrowth parallels related models for KAN, PIN and auxin interaction proposed for the development and polarity establishment of leaf lamina, the embryo

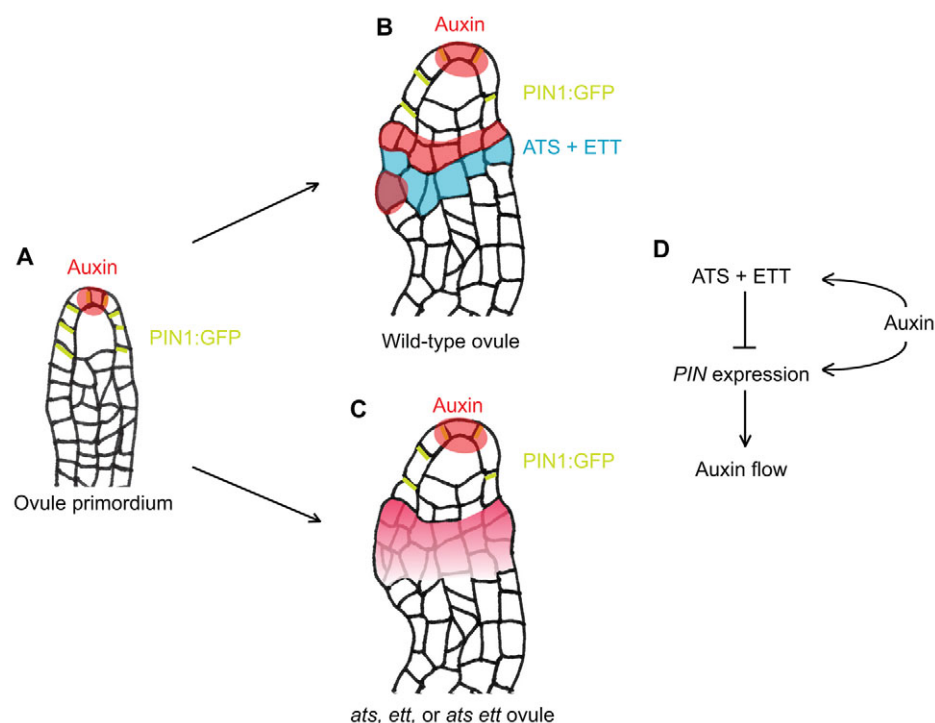


Fig. 4. Model for ATS-ETT action during ovule development.

(A,B) Benkova et al. (Benkova et al., 2003) have shown that PIN1-mediated auxin transport in an ovule primordium initially creates an auxin maximum at the nucellar tip (A) and subsequently two auxin maxima in the chalaza corresponding to the two integument primordia (B). Inner integument initiation coincides with the formation of an active ATS-ETT complex, which we hypothesize restricts the domain of PIN1 and thus enables the formation of the two auxin maxima at the chalaza (B). (C) In the absence of an active ATS-ETT complex (i.e. in *ats* or *ett* mutant ovules) PIN1 fails to be restricted and auxin remains distributed across the chalaza. (D) Formation of ATS-ETT heterodimers restricts PIN1 activity, which controls auxin efflux, while auxin positively regulates both ETT and PIN1 function.

axis, carpels and vascular tissues (Pekker et al., 2005; Izhaki and Bowman, 2007; Ilegems et al., 2010). Our observation that KAN1 also interacts with ETT suggests the possibility that ETT potentiates KAN function in these structures. Further protein-protein interaction tests will evaluate all possible KAN-ARF interactions that might occur in vivo. Although ETT and ARF4 act redundantly in leaves, we were unable to detect interactions between KAN proteins and ARF4 (supplementary material Fig. S1), but it is possible that an in planta interaction requires other factor(s). Thus, it is possible that KAN-ETT protein complexes act differently in organs other than inner integuments, consistent with the distinct evolutionary origins of leaves and the inner integument (Endress, 2011).

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at
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Table S1. Primers used for cloning

Primer	Sequence (5'-3')	Vector	Type
ETT R1	CTAGAGAGCAATGTCTAGCAACATGT	pAA24	Reverse
DK255	CACCATGGAGCTGTTTCCTGCTCAGCC	pAA29	Forward
DK257	TTAGTGAGATCGACCCAGAGTAAACTCAAG	pAA29	Reverse
DK258	CACCATGGAATTTGACTTGAATACTGAGA	pAA30	Forward
DK260	TCAAACCCTAGTGATTGTAGGAGA	pAA30	Reverse
DK240	CaccATGATGATGTTAGAGTCAAGAAACA	pAA34	Forward
DK272	GCACTTGAGAAGGGTTAAATCA	pAA34	Reverse
116ETTfor	CTTCTCGAGATGGGTGGTTTA	pCG54	Forward
116ETTrev	CTTCCC GGGAGAGCAATGTCTAGCAAC	pCG54	Reverse
115ATSfor	CTTCTCGAGATGATGATGTTAGAG	pCG51	Forward
115ATSrev	CTTCCC GGCACTTGAGAAGGGTTAAA	pCG51	Reverse
ATSFNdel	GGAATTCCATATGATGATGATGTTAGAGTC	pDK8	Forward
ATSRNcol	CATGCCATGGTTAGCACTTGAGAAG	pDK8	Reverse
DK244	CACCATGGGTGGTTTAATCGATCTGA	pDK74, pAA24	Forward
ETT R2	GAGAGCAATGTCTAGCAACATGTC	pDK74	Reverse
DK290	ACTCGAGATGATGATGTTAGAGTCAAGAAACA	pDK115	Forward
DK291	AACCCGGGGCACTTGAGAAGGGTTAAATCA	pDK115	Reverse
DK298	ACTCGAGATGGGTGGTTTAATCGATCTGA	pDK116	Forward
DK299	AACCCGGGGAGAGCAATGTCTAGCAACATGTC	pDK116	Reverse
kan15'bam	ATAGGATCCATATGTCTATGGAAGGTGTTTTT	pLMK37/44/46	Forward
kan13'pst	TATCTGCAGTTTCTCGTGCCAATCTGG	pLMK37/44/46	Reverse