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; McBee 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; McBee et al., 2006). ATS plays dual roles during ovule development, providing boundary maintenance and promoting laminar growth of the inner integument (McBee et al., 2006). Similarly, loss of KAN1 and KAN2 disrupts laminar growth of the outer integument (Eshed et al., 2001; McBee 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; McBee 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 ETT (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
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_{ag}-KAN4 (Hawker and Bowman, 2004) by PCR, digested with NdeI/NotI and inserted into pGBK7 (Clontech), creating pDK8 [GAL4 DNA-binding domain (BD)-ATS]. ATS cDNA without the stop codon was amplified from P_{ag}-KAN4 and inserted into pENTR/D-Topo (Invitrogen), creating pAA34. ATS cDNA was amplified from P_{ag}-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-GADT7 (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 a NdeI/XhoI fragment from pDK136 into pGBK7 using NdeI/Sall 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 BamHI/PstI fragment into plMTUMS28 to create pLMK37 and transferred as a BamHI/PstI 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 pCG47 and pCG46, respectively. The cDNAs were inserted into 2X35S-SPYCE or 2X35S-SPYNE vectors (Walter et al., 2004), respectively, as XhoI/XbaI fragments into these sites forming pCG47 and pCG50. The resulting expression cassettes were transferred into pMLBART (Gleave, 1992) as NotI/XhoI fragments and transcribing with T7 RNA polymerase (Promega) and DIG labeling mix (Roche).

RESULTS AND DISCUSSION

Yeast two-hybrid screen with ATS identifies ETTIN

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 (Kerpola,
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 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 3J with 3I; seeds in supplementary material Fig. S2). 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...
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 thus 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.
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 http://dev.biologists.orglookup/suppl/doi:10.1242/dev.067918/-/DC1

References


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