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ABERRANT TESTA SHAPE encodes a KANADI family member, linking polarity determination to separation and growth of Arabidopsis ovule integuments

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Summary

The Arabidopsis *aberrant testa shape* (*ats*) mutant produces a single integument instead of the two integuments seen in wild-type ovules. Cellular anatomy and patterns of marker gene expression indicate that the single integument results from congenital fusion of the two integuments of the wild type. Isolation of the *ATS* locus showed it to encode a member of the KANADI (KAN) family of putative transcription factors, previously referred to as KAN4. *ATS* was expressed at the border between the two integuments at the time of their initiation, with expression later confined to the abaxial layer of the inner integument. In an *inner no outer* (*ino*) mutant background, where an outer integument does not form, the *ats* mutation led to amorphous inner integument growth. The *kan1 kan2* double mutant exhibits a similar amorphous growth of the outer integument without affecting inner integument growth. We hypothesize that ATS and KAN1/KAN2 play similar roles in the specification of polarity in the inner and outer integuments, respectively, that parallel the known roles of KAN proteins in promoting abaxial identity during leaf development. *INO* and other members of the *YABBY* gene family have been hypothesized to have similar parallel roles in outer integument and leaf development. Together, these two hypotheses lead us to propose a model for normal integument growth that also explains the described mutant phenotypes.

Keywords: reproductive development, seed, polarity, dormancy, organ fusion.

Introduction

The ovules of Arabidopsis are typical of those of most angiosperms, comprising an axis with a proximal (close to the point of attachment) funiculus, a distal nucellus (containing the embryo sac), and two lateral sheathing structures – the inner and outer integuments. A variety of genes have been implicated in growth of the integuments, some of which are members of families involved in elaboration of the primary lateral organs of the shoot system: leaves, sepals, petals, stamens and carpels. *INNER NO OUTER* (*INO*) plays a critical role in initiation and extension of the outer integument and is a member of the *YABBY* gene family. Other *YABBY* genes, such as *FILAMENTOUS FLOWER* (*FIL*) and *YABBY3* (*YAB3*), are expressed in primary lateral organs where they play roles in organ polarity and growth (Eshed *et al.*, 2004; Sawa *et al.*, 1999; Siegfried *et al.*, 1999). Members of the *KANADI* (*KAN*) gene family, a subset of the larger *GARP* (from GOLDEN2, ARR-BClass, Par1 proteins) class of transcription factor genes (Riechmann *et al.*, 2000), also play roles in leaf polarity and expansion (Eshed *et al.*, 1999, 2004; Kerstetter *et al.*, 1994). *KAN* genes have roles in ovule development, as indicated by the reduced growth of the outer integument observed in some *kan* mutant combinations (Eshed *et al.*, 2001).

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The *aberrant testa shape* (*ats*) mutation affects only ovule development, where it results in the formation of a single integument in place of the normal two (Léon-Kloosterziel *et al.*, 1994). The single integument was hypothesized to result either from loss of one integument or from a congenital fusion of the inner and outer integuments into a single structure (Léon-Kloosterziel *et al.*, 1994). If the latter model is correct, then the effect of this mutation is similar to a process that has led to the evolution of unitegmy in at least one group of angiosperms (McAbee *et al.*, 2005). Thus, elucidation of the nature and function of *ATS* has the potential to shed light on the processes of organ separation, organ fusion, and ovule evolution.

We have identified the *ATS* locus and report here that it encodes a member of the KANADI family. Polar expression of *ATS* in the inner integument paralleled expression of other *KANADI* family members in leaves. Analyses of additional *ats* alleles showed that the effects of the original *ats* mutant (*ats-1*) were not particular to that allele. A relatively simple model of the relationship between polarity determinants, organ polarity, and growth explained the ovule phenotypes resulting from mutations in *ATS* and other *KANADI* genes.

Results

Ovule development in Arabidopsis *wild type and* ats

Analysis of scanning electron micrographs and plastic sections of wild-type and *ats* (herein referred to as *ats-1*) ovules produced results that were largely consistent with those of previous studies (Léon-Kloosterziel *et al.*, 1994; Robinson-Beers *et al.*, 1992; Schneitz *et al.*, 1995), but provided additional evidence for the single integument of *ats-1* being derived from both inner and outer wild-type integuments. As the megaspore mother cell differentiated in the wild-type ovule, the inner and outer integument primordia emerged through divisions in a ring of dermal cells around the ovule (stage 2-II; stages according to Schneitz *et al.*, 1995) and the gynobasal side (the side oriented toward the base of the gynoecium) of the ovule (stage 2-III-IV; Figures 1a and 2a), respectively. In *ats-1* ovules at a similar stage, a single integument primordium emerged from a region several cells wide (Figures 1b and 2b) that was similar in extent to that giving rise to both integuments in wild type (compare Figure 2a,b). Wild-type integuments grew by anticlinal divisions of the L1 initials (Figures 1c and 2c,e), giving rise to two-layered structures (stages 2-IV and 2-V; Figure 2e). In contrast, *ats-1* integuments grew by both anticlinal and periclinal divisions, resulting in a three- to five-layered structure (Figure 2f) with multiple cell files at its apex (Figure 1d; arrow). In the wild type, the boundary between integuments remained distinct as the outer integument grew over the inner integument (stage 2-V; Figures 1c and 2c,e). In *ats-1* ovules, a clear boundary in the single integument was not evident at any stage (Figures 1d and 2d,f). The single *ats* integument remained shorter at stage 3-II (Figure 2f) than

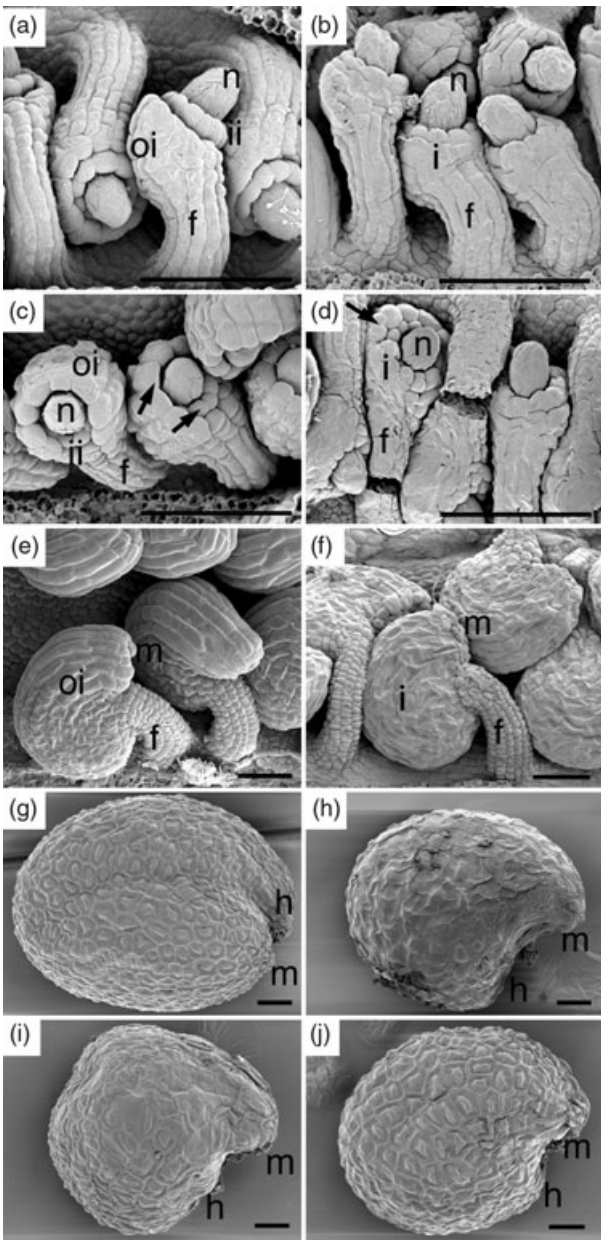

Figure 1. Scanning electron micrographs of developing Arabidopsis wild-type (a, c, e, g) and *aberrant testa shape* (*ats*) (b, d, f, h-j) ovules and seeds. Integument initiation in wild-type (a) and *ats-1* (b) ovules is shown.
(c) In wild-type ovules, one cell file is evident at the tip of each integument (arrows) as the outer integument envelops the inner integument and nucellus. (d) In *ats-1* ovules, two cell files are evident (arrow) in a single integument. Wild-type (e) and *ats-1* (f) ovules appear similar at anthesis. The micropyle and hilum (point of seed detachment) are adjacent in oblong wild-type seeds (g), but separated in roughly heart-shaped *ats-1* (h), *ats-2* (i) and *ats-3* (j) seeds. f, funiculus; h, hilum; i, single integument; ii, inner integument; m, micropyle; n, nucellus; oi, outer integument. Scale bars are 50 μm.

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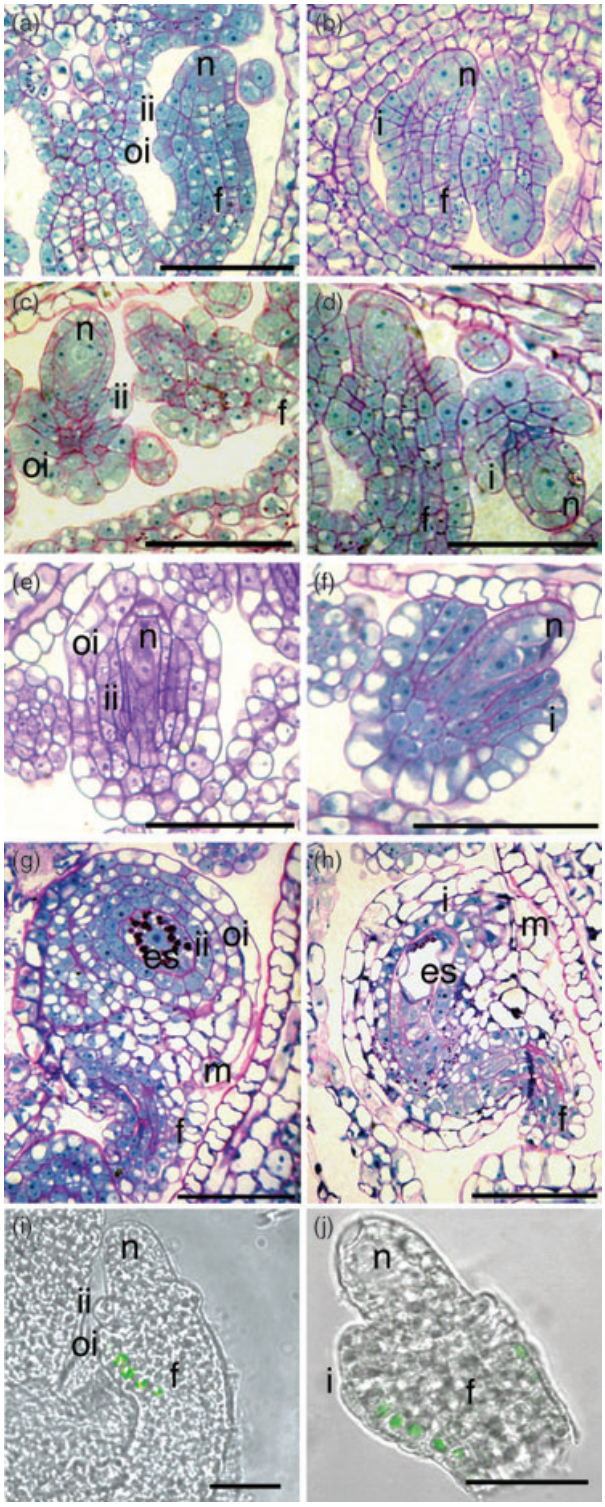
the wild-type outer integument which covered the nucellus and inner integument (Figure 2e).

At anthesis (stage 4-I), wild-type and *ats-1* ovules were similar in external appearance (Figure 1e,f). In the wild type, the cells of the outer integument differed from those of the inner integument, being larger in size and having more prominent vacuoles than the smaller, densely cytoplasmic inner integument cells (Figure 2g). The single *ats-1* integument consisted of three to five cell layers, with the cells in the outermost layers being large and vacuolated (as in the wild-type outer integument) and the innermost cell layers more densely cytoplasmic (as in the wild-type inner integument; Figure 2h). The outer layer of the outer integument is also characterized by the presence of *INO* expression (Balasubramanian and Schneitz, 2000; Meister *et al.*, 2002; Villanueva *et al.*, 1999). In previous studies, *INO* expression was observed in the outer layer of the single integument of *ats* ovules (Balasubramanian and Schneitz, 2002; Villanueva *et al.*, 1999). We confirmed this pattern of expression using a green fluorescent protein (GFP) reporter gene (Figure 2i,j and data not shown).

Following fertilization (stage 4-VI), differences in morphology between wild-type and *ats-1* ovules became evident as the ovules matured into seeds. Wild-type seeds were longer, and the micropyle was uniformly adjacent to the hilum (funiculus scar; Figure 1g). In contrast, *ats-1* seeds were rounder and appeared almost heart-shaped because the micropyle was displaced from the hilum (Figure 1h). The reduced dormancy previously observed for *ats-1* mutants (Léon-Kloosterziel *et al.*, 1994) was also apparent in our studies, where we consistently observed vivipary (germination prior to seed desiccation) for on average one to three of the approximately forty seeds developing in each *ats-1* silique (data not shown). Vivipary was never observed in wild-type siliques.



ATS *encodes a KANADI protein*

Léon-Kloosterziel *et al.* (1994) mapped *ATS* to a region on chromosome 5 between phenotypic markers *transparent testa*, *glabra* and *yellow inflorescence*. Molecular markers were used to further map *ATS*, ultimately localizing it to the

Figure 2. Bright-field microscopy of plastic sections of developing Arabidopsis wild-type (a, c, e, g) and *aberrant testa shape-1* (*ats-1*) ovules (b, d, f, h), and expression analysis of P-INO::INO:GFP (i, j). Initiation of the outer and inner integuments in the wild type (a) and the single integument in *ats* (b) at ovule stage 2-III (gynobasal direction is to the left for both images) is shown. Extension of the outer and inner integuments in wild-type ovules (c) and the single integument in *ats* ovules (d) has begun by ovule stage 2-V. The outer integument envelops the inner integument and nucellus in the wild-type ovule (e), while the single integument of the *ats* ovule (f) still does not cover the nucellus at stage 3-I. At anthesis (stage 4-I), distinct cell layers are evident in the outer and inner integuments of the wild-type ovule (g) and in the single integument of the *ats* ovule (h). Composite images of digital interference contrast and confocal INO:GFP fluorescence were assembled for wild-type (i) and *ats-1* (j) ovules harboring a P-INO::INO:GFP fusion transgene (Meister *et al.*, 2002). The INO:GFP fusion protein accumulates in the nuclei of cells of the abaxial layer of the outer or single integument (respectively) on the gynobasal side of the developing ovules at stage 2-V. es, embryo sac; f, funiculus; i, single integument; ii, inner integument; m, micropyle; n, nucellus; oi, outer integument. Scale bars are 50 μm for (a–h) and 25 μm for (i) and (j).

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region between two newly constructed markers, MDH9C and SNP MJB21-1. There were fourteen annotated genes in this region (At5g42500 through At5g42645) in the databases available at this time (The Arabidopsis Information Resource; http://arabidopsis.org/). We examined the relative levels of expression of the majority of these genes in the gynoecia of the wild type compared with *aintegumenta-4* (*ant-4*, which lacks integuments; Baker *et al.*, 1997) using hybridization to oligonucelotide microarrays. Among the tested genes, only At5g42630 [also referred to as *KANADI4* (*KAN4*); Eshed *et al.*, 2001] exhibited significant differential expression, being 7-fold lower in *ant-4* relative to wild type. This implicated At5g42630 as being expressed in integuments. cDNAs of At5g42630 were isolated from wild-type and *ats-1* plants by reverse transcriptase PCR. Sequence analysis revealed a nonsense mutation in the coding region of the cDNA deriving from *ats-1* (Figure 3). This nonsense mutation introduces a stop codon upstream of the region encoding the conserved GARP domain (Riechmann *et al.*, 2000) found in all KANADI family members (Figure 3; Eshed *et al.*, 2001), implicating this locus as *ATS*.

We obtained two additional lines with different mutations in At5g42630. The TILLING (McCallum *et al.*, 2000) line CS93231, which has a point mutation that results in substitution of a leucine residue for a conserved proline residue near the center of the GARP domain of At5g42630 (Figure 3), was obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State (Columbus, OH, USA). A line that had a T-DNA insertion within the region encoding the GARP domain was identified from an insertion collection (Campisi *et al.*, 1999; Figure 3). Microscopic examination of lines homozygous for either of these mutations revealed ovule and seed phenotypes that could not be distinguished from *ats-1* homozygotes (Figure 1i,j, and data not shown). The correlation of the Ats$^-$ phenotype with three independently identified deleterious mutations in At5g42630 confirmed this locus as *ATS* and showed that ATS (KAN4) is a member of the KANADI family of putative transcription factors (Eshed *et al.*, 2001; Kerstetter *et al.*, 2001). The *TILLING* and insertion mutants were thus designated *ats-2* and *ats-3*, respectively. The short protein product predicted to be produced by the *ats-1* allele lacks most conserved regions of the protein and would likely result in a complete lack of function. *ats-1* is therefore likely a null allele. The similar phenotypes observed in *ats-2* and *ats-3* indicate that they are likely also severe or complete loss-of-function alleles.

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  1 MMMLESRNSM RASNSVPDLS LQISLPNYHA GKPLHGGDRS STSSDSGSSL
                                      Stop(ats-1)
 51 SDLSHENNFF NKPLLSLGFD HHHQRRSNMF QPQIYGRDFK RSSSSMVGLK
                                     L(ats-2)
101 RSIRAPRMRW TSTLHAHFVH AVQLLGGHER ATPKSVLELM NVKDLTLAHV
     Insert(ats-3)
151 KSHLQMYRTV KCTDKGSPGE GKVEKEAEQR IEDNNNNEEA DEGTDTNSPN

201 SSSVQKTQRA SWSSTKEVSR SISTQAYSHL GTTHHTKDNE EKEDTNIHLN

251 LDFTLGRPSW GMEYAEPSSD LTLLKC
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Figure 3. ABERRANT TESTA SHAPE (ATS) amino acid sequence.
The locations of the three described *ats* mutations are indicated by gray highlighting, and their effects on the sequence are indicated above each mutation site. In *ats-1* and *ats-2*, mutations result in nonsense and missense changes, respectively. In *ats-3*, a T-DNA is inserted just upstream of the codon encoding the indicated serine residue. The GARP region conserved in all GARP proteins (Riechmann *et al.*, 2000) is indicated by double underlining, and other motifs reported to be conserved among the four Arabidopsis KANADI proteins (Eshed *et al.*, 2001) are indicated with single underlines.

ATS *is expressed at the boundary between the inner and outer integuments*

ATS (*KAN4*) has previously been shown to be expressed in roots, where it has redundant activity with the other *KANADI* genes (Hawker and Bowman, 2004). The expression pattern of *ATS* during flower development was examined by *in situ* hybridization with the *ATS* cDNA (Hawker and Bowman, 2004). Hybridization to sectioned inflorescences revealed accumulation of *ATS* mRNA only in the ovules and vascular bundles of the carpel walls (Figure 4 and data not shown). In the ovules, mRNA accumulation was first observed with the emergence of the inner integument primordium (stage 2-II) where it was present within and below the proximal part of this structure (Figure 4b). The level of *ATS* mRNA accumulation appeared to increase with the growth of the inner integument and the initiation of the outer integument, but was confined to the abaxial side of the inner integument and the adaxial side of the outer integument (with the funiculus–nucellus line as the axis of the ovule; Figure 4c,d). By stage 2-IV, the *ATS* mRNA was no longer detected in the outer integument and the hybridization signal was confined to the abaxial layer of the inner integument (Figure 4f) in a pattern reminiscent of the abaxial pattern of expression of other *KANADI* genes in leaves (Eshed *et al.*, 2004; Kerstetter *et al.*, 1994). Cross-sections showed that *ATS* mRNA was uniformly distributed around the circumference of the inner integument (Figure 4e). Thus, the pattern of *ATS* expression in ovules correlates precisely with the cells that appear to be most affected by mutations in this gene.

KANADI *loss of function effects on ovule development*

Our determination that *ATS* encodes one of the four members of the KANADI family led us to more closely examine the effects of mutations in other *KANADI* genes on ovule development. Individually, *kan1* and *kan2* mutations have, at most, subtle effects on carpel development and do not appear to affect ovule development (Eshed *et al.*, 1999, 2001). In contrast, in the *kan1-2 kan2-1* double mutant the external structures of the carpel are largely absent (Eshed *et al.*, 2001), but ovules arise from a surface or column of tissue at the center of the flower. These ovules have apparently normal inner integuments, but the usually

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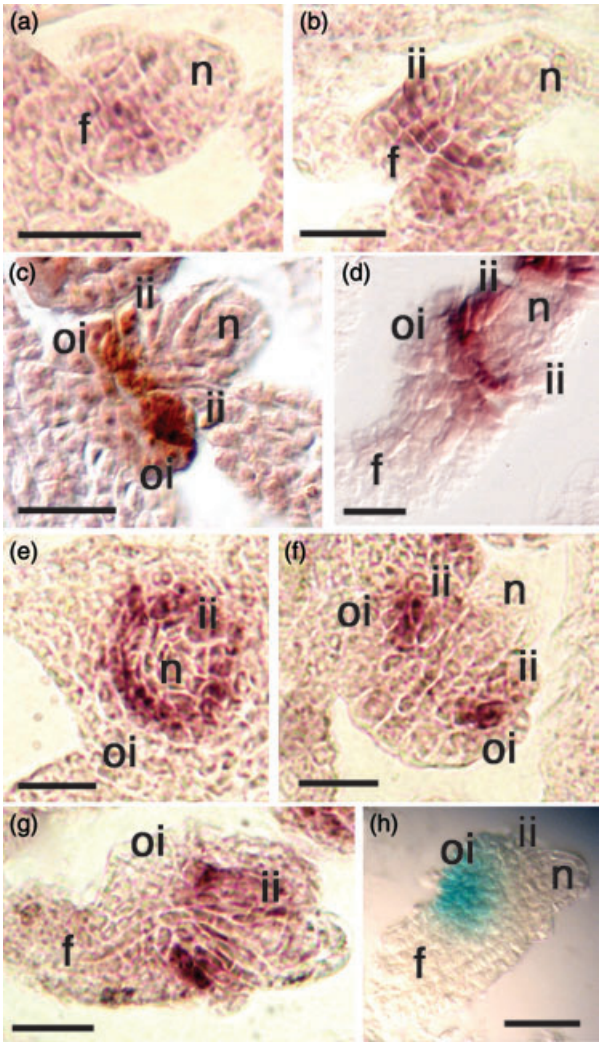

Figure 4. *ABERRANT TESTA SHAPE* (*ATS*) expression during wild-type ovule development and *INNER NO OUTER* (*INO*) transgene expression in the *kanadi* mutant *kan1 kan2*.
(a–g) *In situ* hybridization to ovule sections with *ATS* antisense RNA probe.
(a) At stage 2-I, weak signal is visible in the chalazal region between the funiculus and nucellus.
(b) At stage 2-II, *ATS* hybridization signal is detected on the proximal side of the initiating inner integument.
(c, d) At stage 2-III, signal is visible in abaxial cells of the inner integument and in the adaxial cells of the outer integument.
(e) At stage 2-IV, expression is visualized around the circumference of the entire inner integument.
(f) Also at stage 2-IV, expression appears to become confined to the abaxial layer of the inner integument and is absent from the outer integument.
(g) Expression is still apparent at stage 2-V when it is confined to the proximal part of the abaxial layer of the inner integument.
(h) Histochemical staining for β-glucuronidase activity produced by a P-INO:GUS transgene (Meister *et al.*, 2002) in a plant homozygous for the *kan1-2* and *kan2-1* mutations. Staining is visible in the abaxial region of the amorphous structure that grows in place of the outer integument. f, funiculus; i, single integument; ii, inner integument; n, nucellus; oi, outer integument. Scale bars are 25 μm.

asymmetrical outer integument is replaced by a nearly symmetrical and relatively amorphous collar of tissue (Eshed *et al.*, 2001).

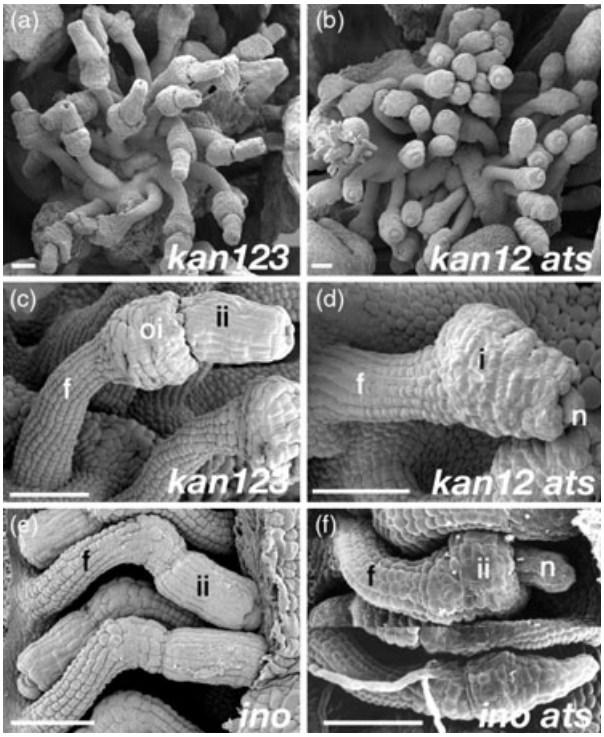

Figure 5. Mutant effects on integument development. Scanning electron microscopy was used to examine ovules of mutant plants at anthesis.
(a, c) The *kan1-2 kan2-1 kan3-1* triple *kanadi* mutant produced ovules from the central part of the flower. These ovules had an apparently normal inner integument, but produced a relatively amorphous growth in place of the outer integument. This phenotype was indistinguishable from that of the *kan1 kan2* double mutant (Eshed *et al.*, 2001).
(b, d). The *kan1-2 kan2-1 ats-3* triple mutant produced a single amorphous structure in place of the two integuments.
(e) *inner no outer-1* (*ino-1*) mutant ovules did not form outer integuments, but the inner integuments developed normally.
(f) In the *ino-1 aberrant testa shape-1* (*ats-1*) double mutant the outer integument was absent and the inner integument was replaced by an amorphous growth of tissue. f, funiculus; i,single integument; ii, inner integument; n, nucellus; oi, outer integument. Scale bars are 50 μm.

We examined a *kan1 kan2 kan3* triple mutant to assess the role of this third gene in carpel and ovule development. We found that flowers of *kan1 kan2 kan3* plants were indistinguishable from those of the *kan1 kan2* double mutant, exhibiting the same absence of external carpel structures and production of central exposed ovules (Figure 5a; Eshed *et al.*, 2001). The ovules of the double and triple mutants were also indistinguishable, with normal inner integuments and amorphous outer integuments (Figure 5c; Eshed *et al.*, 2001). These results indicate that *KAN1* and *KAN2* play a redundant fundamental role in outer integument form and extension, and that *KAN3* does not have overlapping activity.

Flowers of *kan1 kan2 ats* triple mutant plants were similar in structure to those of *kan1 kan2* mutants, but the ovules exhibited a novel phenotype, forming a single amorphous

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structure in place of both integuments (Figure 5b,d). As seen in *ats* single mutants (Figure 2b), growth was initiated in a region that would normally give rise to both integuments but as a single structure, rather than two separate primordia (data not shown). The structure never resolved into the cylindrical laminar structure seen in wild-type ovules or *ats* single mutants, rather growing in all directions to form a collar of tissue that partially covered the nucellus. Thus, in the background of the *kan1 kan2* double mutant, *ats* had two effects – fusion of the integument primordia, and disruption of normal 'laminar/cylindrical' growth of the inner integument, leading to production of an amorphous structure.

Severe *ino* mutations lead to a failure of the outer integument to initiate or grow (Baker *et al.*, 1997; Villanueva *et al.*, 1999; Figure 5e). The *ino* mutant background therefore allows examination of effects of other mutations on the inner integument in the absence of influence of the outer integument. The combination of the *ino-1* and *ats-1* mutations leads to an absence of the outer integument, and a relatively amorphous collar-like structure in place of the inner integument (Figure 5f; Baker *et al.*, 1997). The amorphous inner integument of the *ats ino* mutant is similar in structure to the distal part of the single amorphous integument in the *kan1 kan2 ats* mutant, but appears to have a lesser tendency to grow to cover the nucellus.

The phenotype of the *ats* mutant shows that *ATS* has a role in separation of the integuments. The tested lines with multiple mutations demonstrate that *ATS* also is an essential factor for directing 'laminar' growth of the inner integument. The latter function appears to mirror a similar function for the combination of *KAN1* and *KAN2* in the outer integument.

Eshed *et al.* (2004) showed that loss of KANADI activity in leaves led to a loss of *YABBY* gene expression in these structures. Given the profound effects on ovule development from loss of INO function, we thought it possible that the effects of *kan1-2 kan2-1* mutants on integument growth could be produced through similar reductions in *INO* (a *YABBY* family member) expression. *INO* expression was monitored in the *kan1-2 kan2-1* double mutant using a P-INO::GUS transgene (Meister *et al.*, 2002; Figure 4h). Expression of the transgene was in the abaxial layer of the gynobasal side of the aberrant outer integument (Figure 4h), a pattern similar to that observed in wild-type ovules (Meister *et al.*, 2002). Phenotypic effects of loss of *KAN1 KAN2* function therefore do not appear to result from reduced *INO* expression.

Discussion

ats *mutations result in congenital integument fusion*

On the basis of anatomical studies, Léon-Kloosterziel *et al.* (1994) hypothesized that the single integument of *ats-1* mutants derived either from absence of an integument or from congenital fusion of the two wild-type integuments. Our results provide compelling support for the integument fusion hypothesis. Scanning electron and light microscope analyses showed that at stage 2-III the *ats* integument primordium was much broader than either the inner or the outer wild-type integument primordium and occupied the region from which both integument primordia derive in the wild type. Léon-Kloosterziel *et al.* (1994) observed only three cell layers in the *ats-1* integument at any stage. In contrast, under our growth conditions, the single integument of *ats* ovules consisted of three to five cell layers. In both cases, the single *ats* integument was observed to have more layers than either the inner or the outer integuments throughout its development. In contrast to the wild type, periclinal divisions that added thickness to the single integument often occurred in *ats* ovules in late stages (2-IV–2-V). These extra periclinal divisions and the resulting cell layers suggest that cell division between integument primordia is no longer repressed in *ats* ovules.

Following anthesis (stage 4-I), the outer cell layers of the *ats* integument were large and vacuolated, and the most exterior layer exhibited *INO* expression and columellae formation, all of which are also features of the wild-type outer integument cells. Cells of the inner cell layers of the *ats* integument were smaller and more densely cytoplasmic, with the innermost forming an endothelium – all features of the wild-type inner integument cells. Thus, the *ats* integument included cell types found in both wild-type integuments in the arrangement expected for a congenital fusion.

We conclude that *ats* mutants have a single integument as a result of a congenital fusion of the two integuments and that one role of ATS is to define the boundary that separates these two structures. The expression pattern of *ATS* correlates closely with the location of the division between the two integuments, providing further support for this role in ovule development.

ATS *as a determinant of inner integument polarity*

Examination of the *ats ino* double mutant allowed us to observe the effects of loss of ATS activity in the absence of an outer integument. Here we saw that growth did occur in the region that would normally give rise to the inner integument, but this growth produced an amorphous bulge of tissue, rather than the normal laminar inner integument. This alteration in growth pattern was similar to that produced in the outer integument as a result of loss of function of the *KAN1* and *KAN2* genes. This indicates that *ATS* and the common *KAN1/2* function play similar roles in promoting laminar growth of the inner and outer integuments, respectively. This common function is further supported by the fact that all three genes encode members of the KANADI protein family.

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KANADI family members have been previously shown to play roles in initiation of lateral roots (Hawker and Bowman, 2004) and vascular tissue (Emery *et al.*, 2003), and in the polarity and development of lateral organs of the shoot system (Eshed *et al.*, 1999, 2001, 2004; Kerstetter *et al.*, 2001). *KAN1*, *KAN2*, and *KAN3* are the genes involved in the latter function (Emery *et al.*, 2003; Eshed *et al.*, 2004), and *ATS* (*KAN4*) expression was not observed in these organs (this work). Loss of *KAN1/2/3* function leads to deficient expansion of lateral organ laminar structures, including leaves, petals, sepals, and carpel walls (Eshed *et al.*, 2004). This has been hypothesized to be attributable to a loss of abaxial identity (Eshed *et al.*, 2001, 2004). Juxtaposition of abaxial and adaxial identities is hypothesized to be required to promote lamina formation and expansion in primary lateral organs (Waites and Hudson, 1995; Waites *et al.*, 1998; Figure 6a).

Although they are topologically closed around the circumference of the ovule into a cylinder (inner integument) or hood-shaped structure (outer integument), emergence and growth of the two integuments from the chalaza still represent a laminar pattern. We have previously hypothesized that a juxtaposition of abaxial and adaxial regions is necessary for outer integument growth, with *INO* as an essential abaxial determinant in this structure (Meister *et al.*, 2002; Skinner *et al.*, 2004). Our current results lead us to extend this model to the inner integument and to include members of the *KANADI* gene family as additional abaxial determinants. Such a model, in which *ATS* and *KAN1/2* are abaxial determinants of the inner and outer integuments, respectively, can explain the observed phenotypic effects of mutations in these genes on ovule development (Figure 6).

The expression of *ATS* in the abaxial side of the inner integument supports this model. Furthermore, we have shown that in an *ino* mutant background, where the outer integument is absent, the effect of loss of ATS function is a failure of laminar growth of the inner integument. This failure, and the subsequent growth of an amorphous structure in place of the inner integument, may be analogous to the formation of filamentous structures in place of leaves as a result of loss of polarity in *kan1 kan2 kan3* (Eshed *et al.*, 2004) or dominant *phabulosa* (*phb*) mutants (McConnell *et al.*, 2001). The amorphous inner integument would in this sense be a 'filament' in a ring around the circumference of the ovule primordium, forming as a result of loss of the abaxial identity that is normally provided by the *KANADI* family member *ATS*. Our model further proposes that *KAN1* and *KAN2* redundantly provide a function in the outer integument that is homologous to that provided in the inner integument by *ATS* and also similar to the function of these genes in leaves. The amorphous outer integument formed in *kan1 kan2* mutants would be the result of loss of the abaxial identity provided by these two genes in the outer integument. While *KAN3* acts redundantly in leaves with *KAN1* and *KAN2* (Eshed *et al.*, 2004), *KAN1* and *KAN2* appear to be the only *KANADI* genes functioning in outer integument development.

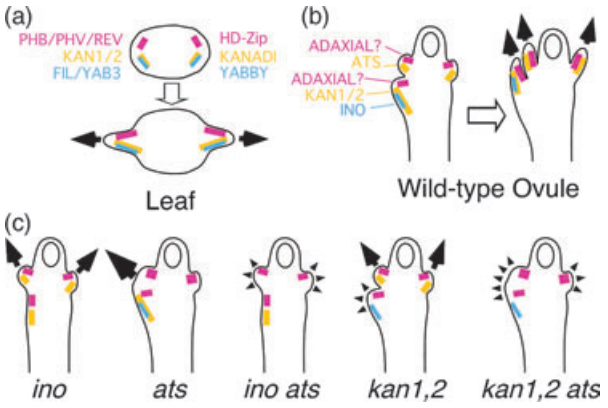

Figure 6. Model for roles of *KANADI* and *YABBY* genes in ovule development. Locations of expression of adaxial factors and abaxial KANADI and YABBY factors are shown in magenta, orange and turquoise, respectively.
(a) Leaf lamina expansion depends on juxtaposition of adaxial and abaxial factors. The *homeodomain-leucine zipper* (*HD-Zip*) genes *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), and *REVOLUTA* (*REV*) are expressed in the adaxial domain of leaf primordia where they promote adaxial identity (Emery *et al.*, 2003; McConnell and Barton, 1998; McConnell *et al.*, 2001). *KANADI* (*KAN1, KAN2* and *KAN3*) and *YABBY* [*FILAMENTOUS FLOWER* (*FIL*) and *YAB3*] genes are expressed abaxially and promote abaxial identity (Eshed *et al.*, 2001, 2004). The point of intersection of the adaxial and abaxial domains determines the location and direction of laminar expansion (arrows).
(b) In wild-type ovules, we hypothesize that as yet unidentified adaxial factors act in juxtaposition with the abaxial factors KANADI [ABERRANT TESTA SHAPE (ATS) in the inner integument and KAN1 and KAN2 in the outer integument] and the YABBY protein INNER NO OUTER (INO; in the outer integument), to define the locations for laminar growth of the two integuments.
(c) Effects of mutations on integument formation. In the *ino* mutant, loss of this abaxial YABBY activity precludes formation of the outer integument. Inner integuments grow normally. In the *ats* mutant, loss of this abaxial KANADI activity in the inner integument leads to extension from a single larger region (large arrow) between the inner integument adaxial region and the next abaxial region defined by *INO* and *KAN1* and *KAN2* at the point of normal origin of the outer integument. This results in the formation of a single integument that is thicker than either of the two wild-type integuments. In the *ats ino* double mutant the outer integument is absent as a result of the *ino* mutation. The absence of KANADI (ATS) activity in the abaxial zone of the inner integument disrupts definition of the location of laminar growth, and amorphous growth (arrowheads) occurs. In the *kan1 kan2* double mutant, normal laminar growth of the inner integument occurs, but the absence of the KANADI function in the abaxial domain of the outer integument leads to amorphous growth (arrowheads) of this structure. In the *kan1 kan2 ats* triple mutant, a single broad zone of growth is defined by the adaxial determinant of the inner integument and the YABBY (INO) determinant in the outer integument. Growth from this region is amorphous (arrowheads) as a result of the absence of KANADI activity.

The model further explains the integument fusion effect of *ats* mutations. In an otherwise wild-type background, loss of ATS function would compromise the specification of abaxial identity in the incipient inner integument. We hypothesize that the specification of inner integument adaxial identity could persist and even expand under these conditions. The space between this region of adaxial identity and the next

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adjacent region of abaxial identity, on the abaxial side of the outer integument precursor, would then be specified as the location of growth. This would lead to growth of a single structure that spans the entire region from which the two integuments would normally arise (Figure 6c).

While our model parallels that outlined for primary lateral organs of plants, there are clear differences in how mutations in *KANADI* and *YABBY* genes affect the growth of such organs in comparison to the integuments. The primary lateral organs only become fully filamentous if both KANADI and YABBY functions are compromised (Eshed *et al.*, 2004). Loss of either function alone results in only a partial reduction in blade outgrowth and partial adaxialization. In contrast, loss of KANADI function in the integuments leads to amorphous growth (analogous to filamentous growth) for either the outer (in the *kan1 kan2* mutant) or inner (in the *ats* mutant) integument. For the outer integument, loss of YABBY function alone in the *ino* mutant had the most severe effect, causing the complete absence of this structure. The more severe effects of the *ino* mutation relative to the *kan1 kan2* mutant indicate that the YABBY function is most critical for growth of the outer integument. The observation that *INO* expression (as measured by a reporter gene) is not altered in *kan1 kan2* mutants shows that the KANADI and YABBY activities function in partial or complete independence in the promotion of growth of the outer integument.

ATS *interactions with other mutations*

Vivian-Smith *et al.* (2001) observed that *ats-1* accentuated the parthenocarpy phenotype of *fruit without fertilization* (*fwf*) mutants. Our observation of *ATS* expression only in the ovules and in carpel vascular bundles indicates that effects of the mutation on the entire carpel wall must be a result of cell non-autonomous functions. One possible mechanism for this is suggested by the reduced dormancy (Léon-Kloosterziel *et al.*, 1994) and vivipary (this work) phenotypes of *ats* seeds. Abscisic acid (ABA) is one substance that has been shown to promote seed dormancy and to be produced by maternal seed tissues (Raz *et al.*, 2001). It is possible that either the adaxial layer of the outer integument or the abaxial layer of the inner integument participates in the production of ABA. Disruption of these layers in the *ats* mutants could reduce ABA production, impairing establishment of seed dormancy. This same reduction in ABA could reduce an ABA- mediated inhibition of carpel expansion, leading to the enhancement of the *fwf* mutant effects.

Based on double mutant and expression analyses, Balasubramanian and Schneitz (2002) proposed a redundant role for *SPOROCYTELESS/NOZZLE* (SPL/NZZ) and *ATS* in maintaining ab–adaxial pattern formation by negative regulation of INO. Early expression of *ATS* is essentially complementary to early expression of *INO*, so ATS could inhibit the expression of *INO* directly or indirectly in concert with the more widely expressed *SPL/NZZ*. Later, *ATS* is expressed only in the abaxial layer of the inner integument, and is therefore not adjacent to the outer integument layer where *INO* is expressed, so a role for *ATS* in maintaining ab–adaxial patterning in the outer integument at these stages is not supported.

Remaining questions

Our model can explain the phenotypes we observe, but it leads to a number of questions that can also serve as tests of the model. While primary components of the adaxial function in lateral organs have been shown to be encoded by members of the *homeodomain-leucine zipper* (*HD-Zip)* gene family (Emery *et al.*, 2003; McConnell and Barton, 1998; McConnell *et al.*, 2001) we do not have data on the nature of adaxial determinants in integuments. The *HD-Zip* genes have been shown to be expressed in integuments (Green *et al.*, 2005; Sieber *et al.*, 2004), with *PHABULOSA* (*PHB*) even being expressed in the adaxial region of the inner integument (Sieber *et al.*, 2004). Ectopic expression of *PHB* in dominant alleles can lead to reduced outer integument growth (McConnell and Barton, 1998), but the significance of this is unclear given that expression of this gene is not normally detected in the outer integument (Sieber *et al.*, 2004). We observed normal ovule development in a line homozygous for loss-of-function mutations in *phb* and *phavoluta* (*phv*), and heterozygous for *revoluta* (D. J. Skinner and C. S. Gasser, unpublished). Minor ovule effects have been reported for a triple mutant of *phb phv* and *corona* (*cna*, the most recently characterized member of this family; Prigge *et al.*, 2005). Thus, if these genes are determinants of adaxial identity in ovules, there must be significant redundancy in their roles. Further examination of combinations of loss-of-function mutants in these genes may reveal activities in integument development, but these studies will be complicated because several combinations of such mutations prevent growth beyond the seedling stage (Prigge *et al.*, 2005).

While ATS in the inner integument is hypothesized to have the same role as KAN1 and KAN2 in the outer integument, no YABBY function to parallel the role of INO in the outer integument has been found for the inner integument. To date we have not observed an integument defect resulting from mutation in any *YABBY* gene besides *INO*, and ovule expression has not been observed by us or reported by others for any *YABBY* gene other than *INO*. The likely separate origins of the inner and outer integuments (Gasser *et al.*, 1998) could mean that different mechanisms act in aspects of growth of these two structures, and no *YABBY* gene may participate in inner integument growth.

Fusion of the two integuments into a single structure is one mechanism through which the unitegmic state has been

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produced in the derived ovules of some angiosperms (McAbee *et al.*, 2005). The genetic mechanisms responsible for this significant change in ovule morphology are not known. The effects of *ats* mutations on Arabidopsis ovule development indicate that *ATS* genes are possible players in this process. Studies on *ATS* orthologs in unitegmic or intermediate species (McAbee *et al.*, 2005) may allow testing of this hypothesis.

Experimental procedures

Plant cultivation

Plants were cultivated as in Baker *et al.* (1997), except that a temperature of 16°C was used to promote robust growth for some examinations. *ats-3* was a kind gift of Lynne Reuber (Mendel Biotechnology, Hayward, CA, USA) and derives from the insertion/enhancer trap collection of Thomas Jack (Campisi *et al.*, 1999). In multiple back- and outcrosses, the *ats-3* allele uniformly cosegregated with the T-DNA insertion in At5g42630. Sources of other mutant lines are referenced in the text.

Microscopy

Arabidopsis tissue was prepared as described previously (Hauser *et al.*, 1998) and examined on a Hitachi (Tokyo, Japan) S3500-N scanning electron microscope. Images were acquired digitally and edited in Photoshop version 7.0 (Adobe, San Jose, CA, USA).

For plastic sectioning, floral tissues were fixed in cold 1.5% glutaraldehyde, 0.3% paraformaldehyde and 0.1% Tween in 0.025 M PIPES, pH 7, overnight and were dehydrated according to Baum and Rost (1996). Tissue was stained with 0.5% saffranin in 95% ethanol to aid visualization during sectioning. Fixed material was infiltrated with Historesin (Leica, Wetzlar, Germany) for 2 weeks at 4°C (Baum and Rost, 1996) and embedded in Historesin plus accelerator in aluminum weigh boats. Embedded tissue was sectioned to 2 μm and stained with the periodic acid-Schiff's (PAS) reagent reaction according to Baum and Rost (1996). Slides were counterstained with 1% aniline blue-black in 7% acetic acid, or with 0.05% toluidine blue in 50 mM Na citrate, pH 5, and 160 mM sorbitol. A Zeiss (Oberkochen, Germany) Axioplan microscope was used for viewing stained sections with bright field illumination. Images were acquired with an MDS290 digital camera (Kodak, New Haven, CT, USA) and edited in Photoshop version 7.0.

Plant transformation with pLMK20 (P-INO::INO:GFP) and confocal microscopy were performed according to Meister *et al.* (2002).

Genetic mapping

ATS was mapped using a population of approximately 200 plants segregating from a cross of *ats-1* [in the Landsberg *erecta* (L*er*) background] with wild-type Columbia-3 plants. *ATS* was first mapped to near the CAPS marker *DFR* (http://www.arabidopis.org, GeneticMarker:1945590) on chromosome five. For more refined mapping we utilized primers based on polymorphisms in the Cereon database which compares the L*er* and Col sequences (Jander *et al.*, 2002). This allowed localization of *ATS* to the region between *MDH9-C* (CER455457) and *MJB21-1* (CER437899; Jander *et al.*, 2002). Genotypes at *MDH9-C* were evaluated through amplification with primers MDH9-CU (ttgttgtggagtaagcttcgg) and MDH9-CL (cctagactaatcccatatcgc) followed by direct electrophoresis. *MJB21-1* was amplified using primers MJB21-1U (caccaaactacattagcctcg) and MJB21-1L (agcccttacgaaagaaaacac) followed by digestion with *Hin*dIII prior to electrophoresis. Tested markers between these two loci cosegregated with *ATS* in the mapping population.

Expression profiling

Total RNA was isolated from wild-type L*er* and *ant-4* (Baker *et al.*, 1997) plants using the Qiagen RNeasy Plant kit (Qiagen, Valencia, CA, USA). Probes were synthesized using the MessageAmp kit, (Ambion, Austin, TX, USA) were hybridized to ATH-1 microarrays (Affymetrix, Santa Clara, CA, USA) using an Affymetrix 450 Fluidics Station, and evaluated on an Affymetrix GeneChip 3000 Scanner according to the manufacturer's instructions.

Array data were processed with the robust multiarray averaging (RMA) algorithm (Irizarry *et al.*, 2003) implemented for BIOCONDUCTOR in the R environment. [http://www.bioconductor.org (Gentleman *et al.*, 2004)]. Fold changes were the ratio of mean RMA values (three independent biological replicates) for arrays probed with wild-type and *ant* samples.

In situ *hybridization*

Tissue fixation, probe preparation and *in situ* hybridization were performed either by the method of Long and Barton (1998) or by a modification (McAbee *et al.*, 2005) of the method of Ferrándiz *et al.* (2000). Digoxigenin-labeled antisense RNA probes were prepared from clones of the entire *ATS* cDNA coding region inserted into either pGEM-7Zf(−) (Promega, Madison, WI, USA) or pLIT28 (New England Biolabs, Beverly, MA, USA) which were linearized with *Bgl*II or *Stu*I, respectively, and transcribed with T7 RNA polymerase. Parallel experiments with other probes (including *INO*) gave different hybridization patterns from those observed for the *ATS* probe, demonstrating that the *ATS* hybridization pattern was not non-specific background (data not shown). The absence of hybridization of the *ATS* probe to vegetative organs showed that the probe did not cross-hybridize with transcripts from other *KAN* genes (as expected given the approximately 55% sequence identity between *ATS* and other *KAN* cDNA sequences).

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