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Expression of ovule and integument-associated genes in reduced ovules of Santalales

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SUMMARY Santalales comprise mainly parasitic plants including mistletoes and sandalwoods. Bitegmic ovules similar to those found in most other angiosperms are seen in many members of the order, but other members exhibit evolutionary reductions to the unitegmic and ategmic conditions. In some mistletoes, extreme reduction has resulted in the absence of emergent ovules such that embryo sacs appear to remain embedded in placental tissues. Three santalalean representatives (*Comandra*, *Santalum*, and *Phoradendron*), displaying unitegmic, and ategmic ovules, were studied. Observed ovule morphologies were consistent with published reports, including *Phoradendron serotinum*, which we interpret as having reduced ategmic ovules, consistent with earlier reports on this species. For further understanding of the nature of the ovule reductions we isolated orthologs of the *Arabidopsis* genes *AINTEGUMENTA* (*ANT*) and *BELL1* (*BEL1*), which are associated with ovule development in this species. We observed ovular expression of *ANT* and *BEL1* in patterns largely resembling those seen in the integumented ovules of *Arabidopsis*. These genes were found to be expressed in the integument of unitegmic ovules and in the surface layers of ategmic ovules, and in some cases, expression of *BEL1* was also observed in the surrounding carpel tissue. We hypothesize that ategmic ovules derive from a fusion of the integuments with the nucellus or that the nucellus has taken on some of the characteristics confined to integuments in ancestral species.

INTRODUCTION

As the precursors to seeds ovules are critical to sexual plant reproduction and to productivity in seed crops. Among angiosperms the most common ovule type is bitegmic in which two integuments cover the nucellus (megasporangium). The broad distribution of bitegmy among angiosperms, especially among the earliest branching groups, led to the conclusion that this is the plesiomorphic state for angiosperms (Gasser et al. 1988; Doyle and Endress 2000). There are, however, groups within the angiosperms that exhibit a reduction from this more common bitegmic state. The order Santalales is one such group that exhibits an especially broad range of ovule types that have been characterized as having two, one, or no integuments (Eames 1977; Bouman 1984).

Santalales comprise mostly hemiparasitic plants (photosynthetic and producing at least some of their own food), although some species of the traditionally circumscribed family Olacaceae are nonparasitic and free-living (Kuijt 1969). Recent molecular phylogenetic work (Malécot and Nickrent 2008) confirmed the polyphyletic nature of "Olacaceae," which has since been reclassified such that it now comprises eight monophyletic families, three of which are basalmost and nonparasitic (Nickrent et al. 2010). Additional molecular phylogenetic work suggests that the holoparasitic (nonphotosynthetic and thus dependent upon host carbohydrates) family Balanophoraceae may be related to, or even a component of, Santalales (Nickrent et al. 2005). Habits range from trees to shrubs, herbs, and aerial parasites such as mistletoes (Vidal-Russell and Nickrent 2008).

Although previous workers have suggested a progressive reduction in ovule complexity when one compares less to more derived members of Santalales, the actual trends are more complex (Fig. 1). "Olacaceae" contains members with two (bitegmic), one (unitegmic), or no (ategmic) integuments on their ovules (Bouman 1984; Sleumer 1984; Malécot et al. 2004). Character evolution in "Olacaceae" is best considered with reference to current family concepts. For example, *Ximenia* (Ximeniaceae) is bitegmic but it occupies a more derived position on the tree than *Strombosia*, *Diogoa* (both Strombosiaceae), and *Erythropalum* (Erythropalaceae) that all have unitegmic ovules. For Olacaceae (in the strict sense), the ategmic condition is present in *Ptychopetalum* and *Dulacia*

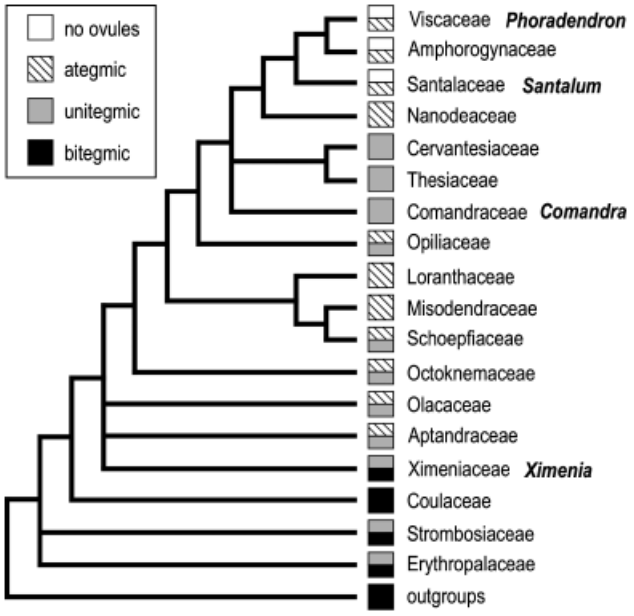


Fig. 1. Ovular integument character states mapped upon a phylogenetic tree depicting relationships among the families of Santalales (after Nickrent et al. 2009). Some families are polymorphic for integument type, that is different genera show different states or a single genus shows both states. Genera utilized in the current study are indicated in bold at right.

whereas various species of *Olax* can be either ategmic or unitegmic (Sleumer 1984).

As with "Olacaceae," "Santalaceae" are also polyphyletic (Der and Nickrent 2008) and have thus been reclassified as six monophyletic families (Nickrent et al. 2010). These families have members that display a range of ovule types, including unitegmic, ategmic, and the lack of actual ovules (Rao 1942; Ram 1957; Bhatnagar and Agarwal 1961; Bouman 1984). In the latter case, ovule reduction has reached an extreme in three lineages that independently evolved the mistletoe habit, that is the eremolepidaceous members of Santalaceae, some Amphorogynaceae, and all Viscaceae. Here the megagametophytes (embryo sacs) can develop directly from a central gynoecial structure referred to as the mamelon (Bhandari and Nanda 1968a, b; Bhandari and Vohra 1983) or "placental nucellar complex" (Ross and Sumner 2005), without the formation of morphologically distinct ovules. The mamelon is subject to different interpretations with some investigators hypothesizing it to be a free-central placenta and others an ovarian papilla (Billings 1933).

The reductions in the ovules of Santalales may have resulted from one of several different mechanisms. For example, an ategmic species could have arisen via loss of the integument(s) or by congenital fusion of the integument(s) with the nucellus. Similarly, the observed absence of morphologically distinct ovules in Viscaceae could have resulted from a failure in ovule development or from the development of ovular tissues within a placenta.

Research on model systems has identified genes such as *AINTEGUMENTA* (*ANT*) and *BELL1* (*BEL1*) that are necessary for ovule development in these species, and whose expression is closely associated with aspects of ovule development (Elliott 1995; Reiser et al. 1995; Skinner et al. 2004). In *Arabidopsis*, *ANT* is expressed in early stages of development of both floral and vegetative organ primordial (Elliott 1995). During ovule development *ANT* is initially expressed in the entire ovule primordium but soon resolves to the chalaza (the central region from which the integuments will emerge) and subsequently specifically marks the developing integuments. *ANT* expression is absent from the nucellus (Elliott 1995). Severe *ant* mutants lack integuments and *ant* mutant ovules thus superficially resemble the ategmic ovules observed in some santalalean species. Within the *Arabidopsis* flower, *BEL1* is expressed only in ovules (Reiser et al. 1995). Initial *BEL1* expression is throughout the ovule primordia, but before integument initiation expression becomes confined to the chalazal region. *BEL1* expression is observed in the integument primorida, but disappears as the integuments mature (Reiser et al. 1995). In strong *bel1* mutants the integuments are replaced by an amorphous collar of tissue (Robinson-Beers et al. 1992; Modrusan et al. 1994). Thus, *ANT* and *BEL1* are critical for integument formation in *Arabidopsis*, and their expression marks the presence of the chalaza and integument primordia. Orthologs of these genes could serve as markers to evaluate the presence of specific tissues in reduced ovules, and alterations in expression of such genes could be causally related to the ovule reductions. In such studies, members of Santalales with bitegmic or unitegmic ovules can be used to evaluate the conservation of expression patterns of the orthologs of these genes. Such conservation would be an indication of conservation of gene function between the model species and Santalales. Conservation or alteration in the patterns of expression of the genes in the more derived/reduced ovules of other santalalean species could aid in interpretation of their derivation.

Members of three santalalean families were chosen to initiate a study on the developmental basis of the ovule reductions using morphological and anatomical methods in combination with expression analysis of *ANT* and *BEL1* orthologs. Studied species include *Comandra umbellata* (a unitegmic member of Comandraceae), *Santalum album* and a hybrid *Santalum* (ategmic members of Santalaceae), and *Phoradendron serotinum* ssp. *macrophyllum* (a member of Viscaceae interpreted as producing embryo sacs within a bilobed mamelon, Bhandari and Vohra 1983, or within highly reduced ategmic ovules, Billings 1933). This study provides the first direct evidence addressing tissue homologies in santalalean ovules undergoing losses and reductions. Such

Table 1. *AINTEGUMENTA* and *BEL1* primers used in this study

Primer	Direction	Sequence
ANT1-1	Forward	5′ GGCGTGTACTACTCCCACATGHSNGTNATGCC 3′
ANT1-2	Forward	5′ GCTCCCTGTGCATCATGGARGSNHT 3′
ANT3-1	Reverse	5′ GATGGACTTCCGGTGCACNRKYTGYTT 3′
ANT3-2	Reverse	5′ CGGTGCACGGGCTGYTTYTGNCC 3′
ANT6-1	Reverse	5′ CCGCCGCAGGTGGSCNAYRWAYTC 3′
ANT6-2	Reverse	5′ CGCCGCAGGTGGGCNAYRWAYTCYT 3′
BEL1A-1	Forward	5′ CGCAGTACATCTCCTCGACCATNGGNTTCCA 3′
BEL1-1	Forward	5′ CCATGAGGGCCATGTCCMGNCAYTTYMG 3′
BEL1-2	Forward	5′ GGCCATGTCCCGGCAYTTYMGNTG 3′
BEL1-E1	Reverse	5′ CGCAGTACATCTCCTCGACCATNGGNTTCCA 3′
BEL3-1	Reverse	5′ TGCCGGGCCAGGATRTGYTTRTC 3′
BEL3-2	Reverse	5′ GGGGTAGGGGTGCAGGAARTGYTCRAA 3′

information will aid in understanding developmental and evolutionary trends occurring throughout Santalales.

MATERIALS AND METHODS

Plant material

P. serotinum ssp. *macrophyllum* was collected on the UC Davis campus (voucher #Gasser DAV) from July 11, 2005 through August 24, 2005. *Ximenia americana* was collected by Carl Weekely and colleagues at the Archbold Biological Station, Lake Placid FL. *C. umbellata* was collected by Kenneth Robertson (University of Illinois at Urbana-Champaign) and Don Gardner in Livingston County, IL (voucher # 282 Don Gardner ILLS). Samples of *S. album* (voucher # L-74.0013 Lyon) and a hybrid *Santalum* (*S. album* female × *S. freycinatianum* male, voucher #L-66.0339 Lyon) were collected by Karen Shigematsu (University of Hawaii, College of Natural Sciences). All tissue from remote sites was delivered via overnight express mail wrapped in wet paper towels.

Microscopy

Scanning electron microscopy (SEM) followed the procedure of Broadhvest et al. (2000). For light microscopy, tissue was prepared according to Baum and Rost (1996) and was then infiltrated with monomer A from the JB-4 embedding kit (Polysciences, Warrington, PA, USA). Tissue was left in the JB-4 resin for 2 weeks with one change. Sections 5 μm in thickness were cut using a MICROM (Walldorf, Germany) microtome and glass knives. Sections were stained with 0.05% Toluidine blue solution in sodium benzoate buffer at pH 4.4 (Feder and O'Brien 1968). A Zeiss (Oberkochen, Germany) Axoplan 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 with Photoshop CS2 (Adobe, San Jose, CA, USA).

Gene cloning and hybridization

Arabidopsis ANT and the most closely related sequence from rice were aligned using Clustal X version 1.82 (Thompson et al. 1997). CODEHOP (Rose et al. 1998) was used to design degenerate primers (Table 1) corresponding to regions conserved between these sequences that differed from paralogous genes. The locations of the primers on the sequences are illustrated in Fig. S1.

Genomic DNA was extracted from a combination of leaves, flowers and stems using Qiagen's DNEasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The degenerate primers (Table 1) were used in PCR reactions with genomic DNA and either Taq polymerase (New England Biolabs, Beverly, MA, USA) or ExTaq (Takara Bio. Inc., Otsu, Japan). The reaction parameters were 94°C 5 min followed by 40 cycles at 94°C 30 sec, 55°C 30 sec, and 72°C 90 sec. The product obtained from the ANT1-1 plus ANT3-1 reaction was diluted 50-fold and a nested PCR reaction was performed using ANT1-2 and ANT3-2. Once an ANT ortholog was obtained from a given species, specific primers were designed to the ANT ortholog and used with the degenerate primers ANT6-1 and ANT6-2 to obtain additional sequence information from the given species. A similar strategy was used for amplifying orthologs of *BEL1* with degenerate primers (Table 1, Fig. S2). The product resulting from the BEL1-A1 with BEL1-E1 reaction was diluted 50-fold and then used in a nested PCR reaction with BEL1-1 and BEL3-1. This product was then diluted 50-fold and used with BEL3-2 and BEL1-2 in another nested PCR reaction.

PCR products were electrophoresed on 1% agarose gels. Bands of the sizes predicted from the corresponding *Arabidopsis* and rice genes were excised from the gel and cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA, USA). Clones were designated pRB18 (*Comandra ANT*), pRB19 (*Comandra BEL1*), pRB17 (*Santalum ANT*), pRB13 (*Santalum BEL1*), pRB10 (*Phoradendron ANT*), and pRB69 (*Phoradendron BEL1*).

RNA was isolated from flowers and floral buds using the Trizol reagent (Invitrogen). The RNA was then reverse transcribed using the Super Script II enzyme (Invitrogen). Following isolation of an internal part of the cDNA in the degenerate PCR, a 3′ RACE PCR protocol (Sambrook and Russell 2001) was used on cDNAs to isolate the 3′ end to provide a longer probe sequence for in situ hybridizations. Clones were designated pRB77 (*Phoradendron ANT*), pRB78 (*Phoradendron ANT*), pRB79 (*Santalum BEL1*),

pRB80 (*Santalum BEL1*), pRB81 (*Santalum BEL1*), pRB82 (*Santalum ANT*), pRB83 (*Comandra ANT*), pRB84 (*Comandra ANT*), pRB85 (*Comandra BEL1*), pRB86 (*Comandra BEL1*), and pRB92 (*Phoradendron BEL1*). New sequences reported in this work were assigned accession numbers FJ542317–FJ542328.

In situ hybridizations were performed as described by McAbee et al. (2005).

Sequence analyses

Databases including GenBank (http://www.ncbi.nlm.nih.gov/BLAST/ version 2.2.10) and The Institute for Genomic Research (TIGR, http://tigrblast.tigr.org/tgi/ running WU-BLAST 2.0) were searched for genes and proteins similar to *ANT* and *BEL1* using the entire protein sequences from *Arabidopsis*. The sequences were aligned using Clustal X version 1.82 (Thompson et al. 1997) to identify conserved regions in the sequences. Utilizing the results from the initial analyses, putative ANT- and BEL1-specific regions were used to search the databases to ensure identification of the most closely related sequences that were added to the aligments. Alignments were manually edited using MacClade 4 (Maddison and Maddison 2000) and SeAl (Rambaut 2004).

PAUP* version 4.0b10 (Swofford 2003) was used for phylogenetic analysis of the edited sequence alignments. Heuristic searches under maximum parsimony (MP) were performed using an adaptation (Hill et al. 2006) of the BLOSUM62 amino acid weighting matrix (Henikoff and Henikoff 1992), 100 random addition replicates, the tree bisection reconnection algorithm, and saving all trees in each replicate. Support for nodes was determined using 1000 bootstrap replicates of MP heuristic searches with five random sequence addition replicates for each resampling. The NEXUS files used for this analysis are available in supporting information.

RESULTS

Ovule morphology in santalalean taxa

The ovules of *C. umbellata* were found to be unitegmic (Fig. 2, A–E) as reported previously (Ram 1957). Ovule development began with the formation of finger-like projections from a free central placenta (Fig. 2A). Shortly afterwards the single integument initiated growth and subsequently covered the nucellus (Fig. 2B). The asymmetric growth of the integument gave the ovule an anatropous shape (Fig. 2, C–E). This process was similar to what had been observed in *Arabidopsis* where asymmetric growth of the outer integument resulted in anatropous morphology (Robinson-Beers et al. 1992). The placenta in *C. umbellata* became twisted as the ovules matured, eventually attaining a curved morphology when ovule development was complete (Fig. 2D).

Ovules of *S. album* and the hybrid *Santalum* (*S. album* female × *S. freycinatianum* male) were ategmic and orthotropous (Fig. 2, F–I) as reported previously (Rao 1942). The morphology and development of ovules of these two taxa were found to be indistinguishable, thus they will hereafter be referred to collectively as "*Santalum*." *Santalum* ovules initiated as finger-like projections that were nearly hemispherical early in development (Fig. 2F) and elongated as they matured, eventually attaining a more cylindrical shape (Fig. 2G). The placenta, which was small when the ovules initiated, elongated, and enlarged as the flower matured (Fig. 2, G–I). The ovules were appressed tightly to the placenta early in development (Fig. 2G) and remained appressed through completion of embryo sac development (not shown). No indication of any morphologically distinct integument was observed at any stage of *Santalum* ovule development.

Flowers of the majority of the members of the Viscaceae have been interpreted as not possessing ovules (Bhandari and Nanda 1968a, b; Bhandari and Vohra 1983). In contrast to the columnar mamelon structures observed in these species, the embryo sacs of *P. serotinum* were observed to develop within two lobes on the flanks of a central gynocial structure and these have been referred to as nucelli or "ategminous" ovules (Billings 1933). Our observations on *P. serotinum* were consistent with those of Billings (1933). The developing carpels of young female flowers of *P. serotinum* formed a column of tissue in the central cavity (Fig. 2, J and K). Using SEM, the interior of ovaries from mature female flowers showed a central raised structure with two protrusions on opposing flanks (Fig. 2, M and N). Examination of sections of ovaries at a similar stage revealed that each of these protrusions contained an embryo sac (Fig. 2L). No evidence of integument formation was observed at any stage of development. Consistent with Billings (1933) we interpret these structures as ategmic ovules. In contrast to *Santalum*, where the ovules reside in obvious cavities within the carpels (Fig. 2, G and I), in *P. serotinum* the carpel walls were tightly appressed to the ovules such that they could appear to be fused (Fig. 2L).

ANT and *BEL1* ortholog identification

To facilitate the synthesis of primers that would preferentially amplify orthologs of *ANT* and *BEL1* we performed preliminary alignment and phylogenetic analysis of related sequences identified from sequence databases. Within their respective gene families, *Arabidopsis ANT* and *BEL1* fell into well-supported clades containing mostly single representatives from other sampled species (not shown).

Consistent with the work of Kim et. al. (2006) on ANT-related proteins, we found several regions near the N-termini that were only conserved among ANT-related proteins (the euANT lineage), and also found additional regions of sequence that were unique to *Arabidopsis* ANT and the most closely related genes/proteins from other species (Fig. S1). Primers derived from these sequences, and additional conserved sequences in the more C-terminal regions (Table 1), were used to amplify sequences from genomic DNA of *X. americana* (a bitegmic santalalean species), *C. umbellata*,

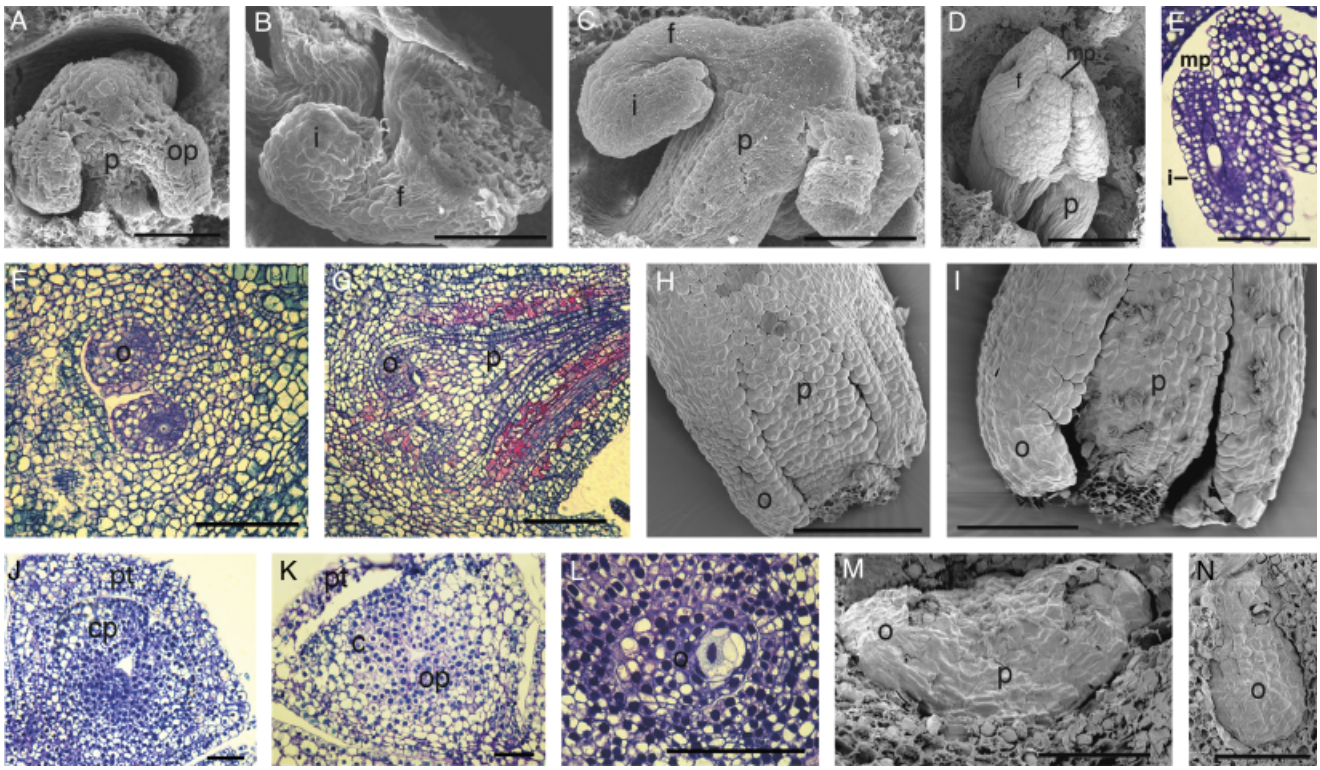
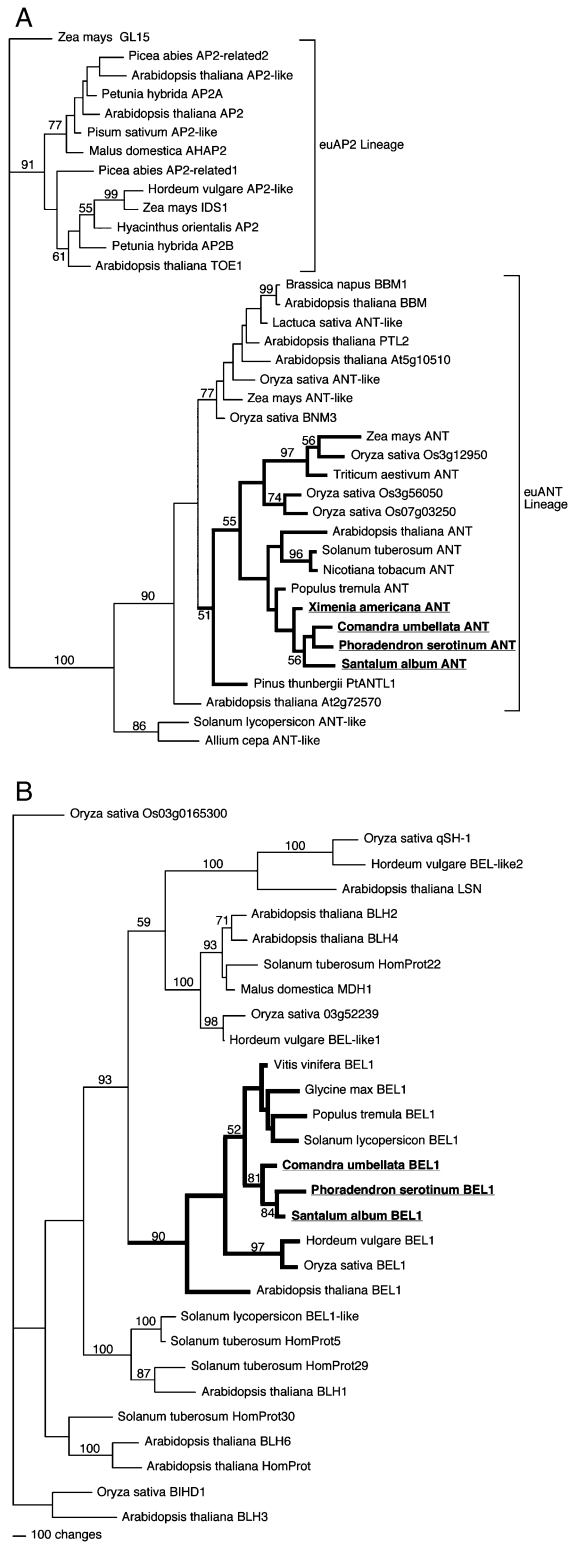


Fig. 2. Morphology of Santalales ovules. (A–E) *Comandra umbellata* ovules. (A–D) Scanning electron micrographs of developing *C. umbellata* ovules. (E) Section through a mature ovule. (A) Young ovule primordia (op) visible as finger-like projections from the placenta (p). (B) The funiculus (f) is visible and the integument (i) has already grown over the nucellus. (C) Ovules attaining their anatropous shape. (D, E) Mature ovules, micropyle (mp). Scale bars are all 100 µm. (F–I) Ovules from *Santalum album* and a hybrid (see text). (F, G) Bright field microscopic images of longitudinal sections through *Santalum* gynoecia. (H, I) SEM images. o, ovules; p, placenta. The scale bars on (F) and (G) are 100 µm, the scale bar on (H) is 200 µm, the scale bar on (I) is 250 µm. (J–N) *Phoradendron serotinum* ovules. (J–L) LM images made by making longitudinal sections through *P. serotinum* flowers. (J) Petals (pt) and carpel primordia (cp) are visible. (K) Ovule primordia (op) are more visible in the carpels (c). (L) A closer view of a section through a mature ovule (o), which is slightly delineated from surrounding carpellary tissue by a small space. (M, N) SEM images showing the ovules of *P. serotinum* made by making a cross section through the base of the female flower. Only one ovule (o) is visible (N), while both ovules are visible (M) along with the placenta (p). The scale bars in all images are 100 µm.

S. album, and *P. serotinum*. Additional sequence was subsequently obtained from amplification of cDNAs.

A MP search was performed on the protein sequences derived from sequences amplified with the ANT primers that were aligned with the previously identified ANT-like sequences, additional closely related sequences, and other representative sequences from the broader APETALA 2 (AP2) family. The dataset was limited to a combination of regions that aligned well among all sequences and some regions that aligned well only within subgroups of the sequences. One hundred random addition replicates of a heuristic search under MP produced a single shortest tree that resolved the "euAP2" and "euANT" lineages of Kim et al. (2006) and Floyd and Bowman (2007) with strong bootstrap support (Fig. 3A). As observed by Floyd and Bowman (2007) and Yamada et al. (2008) we additionally resolved within the euANT lineage a clade including *Arabidopsis* ANT (the putative clade of ANT orthologs corresponding to the "A" clade of Floyd and Bowman, 2007 and the "ANT (sensu stricto)" clade of Yamada et al., 2008) and a clade of proteins most closely related to *Arabidopsis* BABY BOOM (BBM, the "B" clade of Floyd and Bowman (2007) and the "BBM/PLT" clade of Yamada et al. (2008)) with significant bootstrap support (Fig. 3A). Our newly isolated ANT-related santalalean sequences were embedded within the grouping of eudicot proteins in the clade of putative ANT orthologs (Fig. 3A). This analysis indicated that we had isolated orthologs of *Arabidopsis* ANT from the santalalean species.

Similar methods were used to identify regions unique to *BEL1* orthologs (Fig. S2) and these were used to design primers (Table 1) to amplify sequences from *C. umbellata*, *S. album*, and *P. serotinum*. BEL1 full-length sequences could not be unambiguously aligned, thus a smaller portion of 301 aligned positions was used for phylogenetic analysis. Together with other putative BEL1 orthologs from various monocot and dicot species identified in database searches, these new



sequences formed a clade with significant bootstrap support (Fig. 3B). The three santalalean sequences were monophyletic with significant bootstrap support.

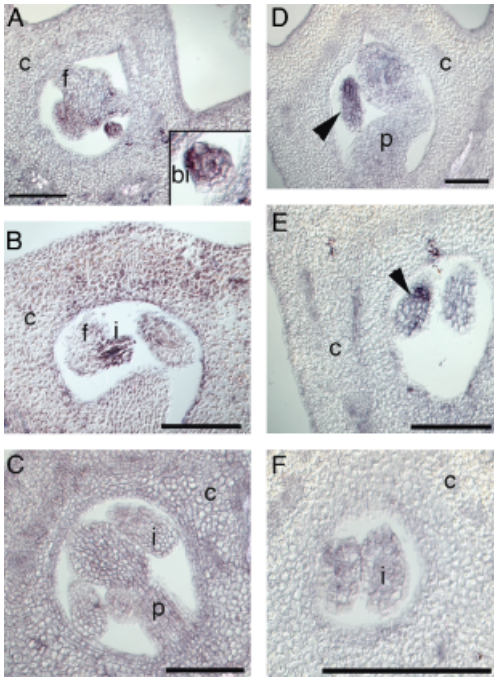
Fig. 3. Relationships of identified genes. (A) The single shortest tree of ANT-related sequences resulting from 100 replications of a random addition heuristic search under maximum parsimony (recovered in 22/100 replicates, length 9043 in arbitrary units; excluding uninformative characters consistency index, 0.6935; homoplasy index, 0.3065; retention index, 0.8279). Numbers adjacent to nodes indicate support from bootstrap analysis. Santalalean sequences are bold and underlined and the putative clade of ANT orthologs is in thicker lines. The euANT and euAP2 clades of Kim et al. (2006) and Floyd and Bowman (2007) are indicated. (B) The single shortest tree of BEL1-related sequences resulting from 100 replications of a random addition heuristic search under maximum parsimony (recovered in 69/100 replicates, length 13,082 in arbitrary units; excluding uninformative characters consistency index, 0.6749; homoplasy index, 0.3251; retention index, 0.6917). Numbers adjacent to nodes indicate support from bootstrap analysis. Santalalean sequences are bold and underlined and the putative clade of BEL1 orthologs is in thicker lines.

Fig. 4. *Comandra umbellata* in situ hybridizations. (A–C) In situ hybridizations with *C. umbellata ANT* anti-sense and sense probes on *C. umbellata* ovules visualized using differential interference contrast microscopy. (A, B) Hybridizations with anti-sense probe. (C) Hybridized with a sense strand probe. (A) Signal is detected in the base of the integument (bi, see enlargement of ovule section in inset for detail) but is absent from the funiculus (f) and carpel wall (c). (B) A longitudinal section through an ovule cutting through the outermost layers of the integument (i) where hybridization is visible. No signal was observed in the integuments of ovules hybridized with the sense probe (C). The scale bars are all 100 μm. (D–F) In situ hybridizations with *C. umbellata BEL1* anti-sense and sense probes on *C. umbellata* ovules. (D, E) Anti-sense probes. (F) Sense probe. (D, E) Hybridization is visible in the integument (arrowheads) of developing ovules. No hybridization is detectable in the integuments of ovules hybridized with the sense probe (F). bi, base of integument; i, integument; p, placenta; f, funiculus; c, carpel wall. The scale bars are all 100 μm.

Expression patterns of *ANT* and *BEL1* in santalalean ovules

ANT mRNA was detected by in situ hybridization in *C. umbellata* ovules in young, developing integuments as well as older, more fully formed integuments (Fig. 4, A and B). A similar pattern was observed for in situ hybridizations with the *BEL1* probe (Fig. 4, D and E).

S. album and the *Santalum* hybrid showed identical staining patterns with an *ANT* probe. The ategmic *Santalum* ovules exhibited *ANT* expression in their distal regions (Fig. 5, A–C). The expression of *ANT* decreased in older ovules of *Santalum*, but because there are no discernible integuments, it was difficult to assess the stage of ovule development at which expression was first detected. *BEL1* expression was also observed in the distal region of the ovules of *Santalum* (Fig. 5, D–F) in a pattern very similar to that observed for *ANT*. *BEL1* hybridization was also detected in the carpel tissue surrounding the ovules.



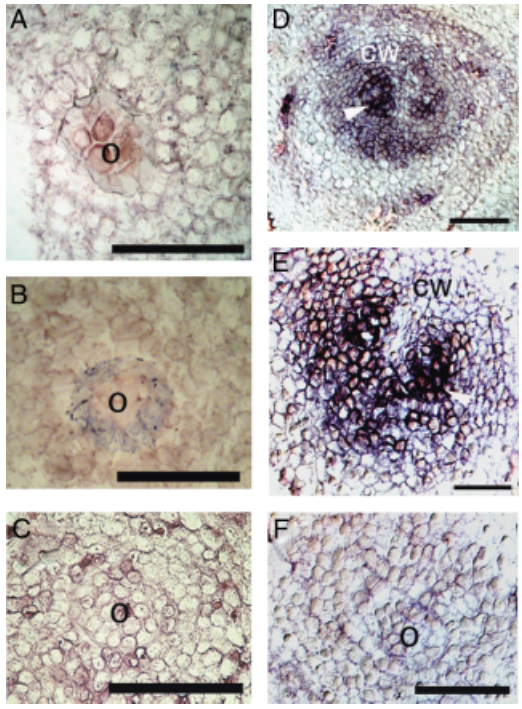


Fig. 6. *Phoradendron serotinum* in situ hybridizations. (A–C) In situ hybridizations of *P. serotinum* ovules with *P. serotinum ANT* anti-sense and sense probes. (A, B) Hybridized with anti-sense probes, (C) Hybridized with a sense probe and visualized with LM. Expression was detected in the outer layer of the ovule as blue staining (A) and red/purple (B). No hybridization was detected with the sense probe (C). o, ovule. The scale bars are all 100 μm. (D–F) In situ hybridizations of *P. serotinum* ovules with *P. serotinum BEL1* anti-sense and sense probes visualized with differential interference contrast microscopy. (D, E) Hybridizations with anti-sense probes. (F) Hybridized with a sense probe. (D, E) Expression was detected in the ovule (o, arrows) and also in the carpel wall (cw) surrounding the ovules. The scale bars are all 100 μm.

The ategmic ovules of *P. serotinum* also exhibited *ANT* expression in a jacket of cells in the outer layer of the ovules (Fig. 6, A and B). *BEL1* expression in *P. serotinum* was also observed in the outer layers of the ovule (Fig. 6, D and E)

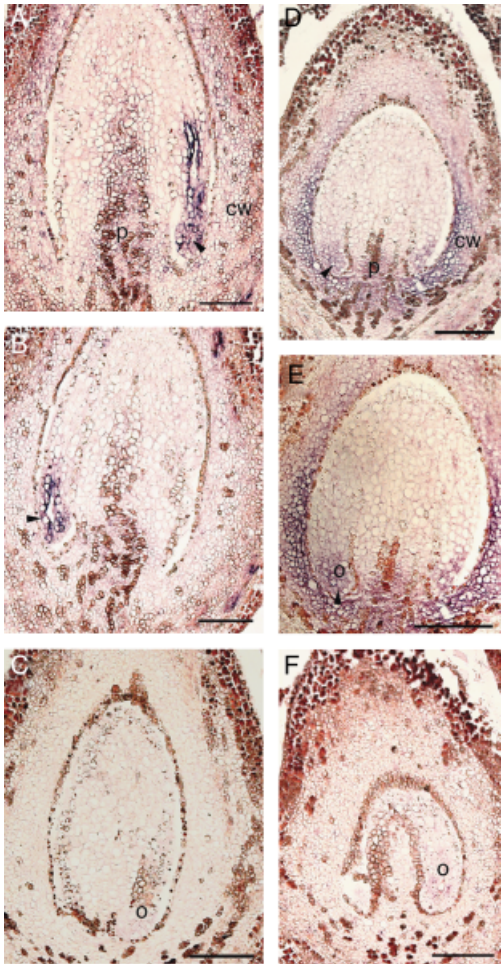


Fig. 5. *Santalum album* in situ hybridizations (A–C) in situ hybridization of *S. album* ovules with *S. album ANT* anti-sense and sense probes visualized with differential interference contrast microscopy. (A, B) Hybridizations with anti-sense probes. (C) A sense probe hybridization. (A, B) Expression was detected primarily in the micropylar end of the ovule (arrows) and also to a lesser extent in the carpel wall surrounding the ovules. No expression was detected with the sense probe (C). o, ovule; p, placenta. (D, E) In situ hybridization of *S. album* ovules with *S. album BEL1* anti-sense and sense probes. (D, E) Hybridized with anti-sense probes. (F) Hybridized with a sense probe. (D, E) Expression was detected in the distal end of the ovule (o, at arrow) and also in the carpel wall (cw) surrounding the ovules. No expression was detected with the sense probe (F). The scale bars are all 100 μm.

similar to the expression of *ANT*. As was the case with *BEL1* in *Santalum*, expression was also observed in the carpel tissue surrounding the ovules in *P. serotinum*.

DISCUSSION

Ovule morphology

The ovule morphologies of *C. umbellata* and *S. album* were found to be as described previously. Prior literature has included a variety of interpretations of the bilobed structure at the center of the gynoecium of *P. serotinum*. Billings (1933) interpreted the lobes as nucelli or ovules lacking integuments. In more recent literature the structure has been described as a bilobed mamelon (Bhandari and Vohra 1983) seemingly to group it with the unitary, unlobed structures in which embryo sacs form in other members of Viscaceae (Bhandari and Nanda 1968a, b; Bhandari and Vohra 1983). We observed a single structure within the gynoecium with two clear lobes, each containing an embryo sac, prompting us to agree with Billings (1933) and interpret these as reduced, ategmic ovules. Topologically this entire structure is very similar in appearance to the structure in *S. album*, which has two ovules on opposite sides of the free-central placenta. The smaller, less defined projections on the placenta of *P. serotinum* could be a reduced version of the larger, more defined pendulous ovules of species like *S. album*. This reductional trend could continue in other Viscaceae where the central gynoecial structure (mamelon or placental–nucellar complex of Ross and Sumner 2005]) that contains the embryo sacs lacks lobes entirely.

Ovule regulatory gene expression in Santalales integuments, ategmic ovules, and carpels

ANT- and BEL1-related sequences were isolated from santalalean species by degenerate PCR. Phylogenetic analysis showed that these sequences partitioned into clades that included only ANT or BEL1 (respectively) as representatives from the *Arabidopsis* genome/proteome. These clades additionally included genes from other eudicot and from monocot species. The santalalean sequences occurred together in a clade in both analyses. These are the results that would be expected if the clades represented groupings of orthologous sequences. In all cases our analysis was consistent with prior analyses (Kim et al. 2006; Floyd and Bowman 2007) but appeared to give superior resolution of these putative clades of orthologs due to our inclusion of a larger number of sequences closely related to ANT and BEL1. On the basis of these observations we conclude that we have isolated santalalean orthologs of ANT and BEL1. While only single *ANT* and *BEL1* genes were identified for each from the three santalalean species, we cannot rule out the possibility that additional co-orthologs exist. The existence of such hypothetical genes would not alter the conclusions drawn in this work that rely specifically on positive observations of expression for the identified genes.

In situ hybridizations performed on *C. umbellata* with orthologs of *ANT* and *BEL1* genes revealed that the two genes are expressed in ovules in patterns that have similarities to those observed in *Arabidopsis* (Reiser et al. 1995; Schneitz et al. 1998) and the unitegmic asterid *Nicotiana tabacum* (Rieu et al. 2005). In these cases, *ANT* expression exists in young as well as mature integuments. These patterns are consistent with possible conservation of *ANT* and *BEL1* functions between *Arabidopsis* and *C. umbellata*.

No published reports for *BEL1* ortholog expression in species other than *Arabidopsis* were found. *MDH1*, a homeodomain protein from *Malus domestica* that is similar to *BEL1*, does not appear to be an ortholog (Dong YH 2000) (Fig. 3B) and is also expressed in ovules; however, its expression is not limited to the integuments and is found throughout the ovule.

Before performing our current work we hypothesized that in situ hybridizations performed on the ategmic ovules of *S. album* and *P. serotinum* with their respective *ANT* and *BEL1* orthologs could have three different outcomes. No hybridization could be observed, and this would indicate that the ategmic ovules were truly ategmic where the entire program of integument development was lost. Hybridization could be observed only at the base of the ovule, indicating that integuments were initiated in the ategmic ovules but were not maintained. Hybridization observed in more of the ovule than just the base would indicate that remnants of the integuments are present but were now fused with the nucellus.

The in situ hybridizations performed on *S. album* and *P. serotinum* showed hybridization with *ANT* and *BEL1* at the distal end or surface of the ovules. *BEL1* also hybridized to the carpel tissue surrounding the ovule. These results suggest the possibility that these species have a remnant of at least one integument that has fused with the nucellus. Thus, this integument tissue would surround the nucellus and embryo sac, as in an ovule with a detached integument. This model suggests that what is absent in ovules of *S. album* and *P. serotinum* is not the integument, but rather is the fissure separating the integument from the nucellus. In principle this is similar to a situation in *Impatiens* where it is proposed that the two integuments have fused into a single structure (McAbee, Kuzoff, and Gasser 2005). The observed hybridization with carpel tissue in *S. album* and *P. serotinum* could be interpreted to indicate that concomitant with ovule reduction, the carpel has acquired some aspects of integument identity. If this is the case, then this process may have proceeded further in *P. serotinum* than in *S. album* because in the former the ovules are even more reduced. A logical end point of this reduction process would be the complete loss of ovular tissues with the carpel taking on all functions of the seed coat. This is observed in the highly reduced gynoecia of members of Balanophoraceae where ovular structures are completely

absent and the embryo sac forms directly within the reduced carpel (Bouman 1984; Endress 1994).

One of the objectives of this study was to test candidate genes for involvement in the reductions of the ovules in Santalales. However, the results of this study indicate that while there has been significant morphological reduction in the ovules, this reduction is not associated with absence of expression of genes normally associated with integument development. In all cases expression of *ANT* and *BEL1* was found in the ovules, thus suggesting that all studied species at least initiate portions of an integument developmental program. Reductions in *ANT* and *BEL1* expression do not appear to be the primary cause of the reduction of the integument(s), rather it appears more likely that steps following expression of these two genes are interrupted. Studies concentrating on genes expressed later than *ANT* and *BEL1* (Gasser et al. 1998; Skinner, Hill, and Gasser 2004) may further illuminate the nature of ovular reductions in Santalales.

The results of our expression studies of *ANT* and *BEL1* on santalalean ovules could be interpreted differently given that *ANT* is expressed in all developing primordia except roots (Elliott 1995) and may be merely a marker for ovule growth. In *Arabidopsis*, early in the development of the ovules, *ANT* expression is associated with ovule growth; however, this expression stops early in primordial expansion and resolves to the integuments. In addition, *BEL1* is not associated with growth in general, but appears to be more specific to the integuments. Together, the most parsimonious hypothesis is that integument tissue is present and is fused with the nucellus.

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REFERENCES

Baum, S. F., and Rost, T. L. 1996. Root apical organization in *Arabidopsis thaliana* 1. Root cap and protoderm. *Protoplasma* 192: 178–188.

Bhandari, N. N., and Nanda, K. 1968a. Studies in the Viscaceae II. A reinvestigation of the female gametophyte of *Arceuthobium ouglasii*. *Am. J. Bot*. 55: 1028–1030.

Bhandari, N. N., and Nanda, K. 1968b. Studies in Viscaceae. I. Morphology and embryology of the Indian dwarf mistletoe- *Arceuthobium minutissimum*. *Phytomorphology* 18: 435–450.

Bhandari, N. N., and Vohra, S. C. A. 1983. Embryology and affinities of Viscaceae. In M. Calder and P. Bernhardt (eds.). *The Biology of Mistletoes*. Academic Press, Sydney, pp. 69–86.

Bhatnagar, S. P., and Agarwal, S. 1961. Morphological and embryological studies in the family Santalaceae—VI. Thesium L. *Phytomorphology* 10: 273–282.

Billings, F. H. 1933. Development of the embryo-sac in *Phoradendron*. *Ann. Bot*. 47: 261–278.

Bouman, F. 1984. The ovule. In B. M. Johri (ed.). *Embryology of the Angiosperms*. Springer-Verlag, New York, pp. 123–157.

Broadhvest, J., Baker, S. C., and Gasser, C. S. 2000. *SHORT INTEGUMENTS 2* promotes growth during Arabidopsis reproductive development. *Genetics* 155: 895–907.

Der, J. P., and Nickrent, D. L. 2008. A molecular phylogeny of Santalaceae (Santalales). *Syst. Bot*. 33: 107–116.

Dong YH, Y. J., Atkinson, R. G., Putterill, J. J., Morris, B. A., and Gardner, R. C. 2000. MDH1: an apple homeobox gene belonging to the BEL1 family. *Plant Mol. Biol*. 42: 623–633.

Doyle, J. A., and Endress, P. K. 2000. Morphological phylogenetic analysis of basal angiosperms: comparison and combination with molecular data. *Int. J. Plant Sci*. 161: S121–S153.

Eames, A. J. 1977. The ovule. In E. Robert (ed.). *Morphology of the Angiosperms*. Krieger Publishing Company Inc, Huntington, NY, pp. 256–289.

Elliott, R. C., Betzner, A. S., Huttner, E., Oakes, M. P., Tucker, W. Q. J., Gerentes, D., Perez, P., and Smyth., D. R. 1995. AINTEGUMENTA, an APETALA2-like gene of *Arabidopsis* with pleiotropic roles in ovule development and floral organ growth. *Plant Cell* 8: 155–168.

Endress, P. K. 1994. *Diversity and Evolutionary Biology of Tropical Flowers, Cambridge Tropical Biology Series*. Cambridge University Press, Cambridge England, UK.

Feder, N., and O'Brien, T. P. 1968. Plant microtechnique: some principles and new methods. *Am. J. Bot*. 55: 123–142.

Floyd, S. K., and Bowman, J. L. 2007. The ancestral developmental tool kit of land plants. *Int. J. Plant Sci*. 168: 1–35.

Gasser, C. S., Broadhvest, J., and Hauser, B. A. 1998. Genetic analysis of ovule development. *Ann. Rev. Plant Physiol. Plant Mol. Biol*. 49: 1–24.

Gasser, C. S., Winter, J. A., Hironaka, C. M., and Shah, D. M. 1988. Structure, expression, and evolution of the 5-enolpyruvylshikimate-3-phosphate synthase genes of petunia and tomato. *J. Biol. Chem*. 263: 4280–4289.

Henikoff, S., and Henikoff, J. G. 1992. Amino acid substitution matrices from protein blocks. *Proc. Natl. Acad. Sci. USA* 89: 10915–10919.

Hill, T. A., Broadhvest, J., Kuzoff, R. K., and Gasser, C. S. 2006. *Arabidopsis* SHORT INTEGUMENTS 2 is a mitochondrial DAR GTPase. *Genetics* 174: 707–718.

Kim, S., Soltis, P. S., Wall, K., and Soltis, D. E. 2006. Phylogeny and domain evolution in the *APETALA2*-like gene family. *Mol. Biol. Evol*. 23: 107–120.

Kuijt, J. 1969. *The Biology of Parasitic Flowering Plants*. University of California Press, Berkeley.

Maddison, D. R., and Maddison, W. P. 2000. *MacClade 4: Analysis of Phylogeny and Character Evolution*. Sinauer Associates, Sunderland, MA.

Malécot, V., and Nickrent, D. L. 2008. Molecular phylogenetic relationships of Olacaceae and related Santalales. *Syst. Bot*. 33: 97–106.

Malécot, V., Nickrent, D. L., Baas, P., van den Oever, L., and Lobreau-Callen, D. 2004. A morphological cladistic analysis of Olacaceae. *Syst. Bot*. 29: 569–586.

McAbee, J. M., Kuzoff, R. K., and Gasser, C. S. 2005. Mechanisms of derived unitegmy among *Impatiens* species. *Plant Cell* 17: 1674–1684.

Modrusan, Z., Reiser, L., Feldmann, K. A., Fischer, R. L., and Haughn, G. W. 1994. Homeotic transformation of ovules into carpel-like structures in Arabidopsis. *Plant Cell* 6: 333–349.

Nickrent, D. L., Der, J. P., and Anderson, F. E. 2005. Discovery of the photosynthetic relatives of the "Maltese mushroom" *Cynomorium*. *BMC Evol. Biol*. 5: 38. DOI: 10.1186/1471-2148-5-38.

Nickrent, D. L., Malécot, V., Vidal-Russell, R., and Der, J. P. 2010. A revised classification of Santalales. *Taxon* (in press).

Ram, M. 1957. Morphological and embryological studies in the family Santalaceae. I—*Comandra umbellata* [L] Nutt. *Phytomorphology* 7: 24–35.

Rambaut, A. 2004. *Se-Al Sequence Alignment Editor, Version 2.0 a11*. Department of Zoology, University of Oxford, Oxford, UK.

Rao, L. N. 1942. Studies in the Santalaceae. *Ann. Bot*. 6: 151–175.

Reiser, L., Modrusan, Z., Margossian, L., Samach, A., Ohad, N., Haughn, G. W., and Fischer, R. L. 1995. The BELL1 gene encodes a homeodomain protein involved In pattern formation in the *Arabidopsis* ovule primordium. *Cell* 83: 735–742.

Rieu, I., Bots, M., Mariani, C., and Weterings, K. A. P. 2005. Isolation and expression analysis of a tobacco *AINTEGUMENTA* ortholog (*NtANTL*). *Plant Cell Physiol*. 46: 803–805.

Robinson-Beers, K., Pruitt, R. E., and Gasser, C. S. 1992. Ovule development in wild-type Arabidopsis and two female-sterile mutants. *Plant Cell* 4: 1237–1249.

Rose, T. M., Schultz, E. R., Henikoff, J. G., Pietrokovski, S., McCallum, C. M., and Henikoff, S. 1998. Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly related sequences. *Nucleic Acids Res*. 26: 1628–1635.

Ross, C. M., and Sumner, M. J. 2005. Early embryo and endosperm development in the dwarf mistletoe *Arceuthobium americanum*. *Int. J. Plant Sci*. 166: 901–907.

Sambrook, J. S., and Russell, D. W. 2001. *Molecular Cloning: A Laboratory Manual*. 3rd Ed. 3 Vols. Cold Spring Harbor Laboratory Press, Woodbury, NY.

Schneitz, K., Baker, S. C., Gasser, C. S., and Redweik, A. 1998. Pattern formation and growth during floral organogenesis: *HUELLENLOS* and *AINTEGUMENTA* are required for the formation of the proximal region of the ovule primordium in *Arabidopsis thaliana*. *Development* 125: 2555–2563.

Skinner, D. J., Hill, T. A., and Gasser, C. S. 2004. Regulation of ovule development. *Plant Cell* 16: S32–S45.

Sleumer, H. O. 1984. Olacaceae. *Flora Neotropica*, Monograph number 38.

Swofford, D. L. 2003. *PAUP*: Phylogenetic Analysis Using Parsominy (and Other Methods), Version 4.0*. Sinauer Associates, Sunderland, MA.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The CLUSTAL*X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*. 25: 4876–4882.

Vidal-Russell, R., and Nickrent, D. L. 2008. The first mistletoes: origins of aerial parasitism in Santalales. *Mol. Phylogenet. Evol*. 47: 523–537.

Yamada, T., Hirayama, Y., Imaichi, R., and Kato, M. 2008. AINTEGUMENTA homolog expression in *Gnetum* (gymnosperms) and implications for the evolution of ovulate axes in seed plants. *Evol. Dev*. 10: 280–287.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Tables S1 and S2. GenBank accession numbers of sequences used in phylogenetic analysis of ANT- and BEL1-related proteins (Fig. 2).

Fig. S1. Partial alignment of ANT sequences used to design gene isolation primers in this study. The regions underlined in red appear to be specific to ANT orthologs and were used to design degenerate primers used in the study. The euANT3 region (PKLEDFLG) is underlined in blue, euANT4 (TFGQR) is underlined in green and euANT1 (NSC[K/R][K/R]EGQ[T/S]R) is underlined in orange (Kim et al. 2006).

Fig. S2. Partial alignment of BEL1 sequences used to design gene isolation primers in this study. The red arrows represent the locations in which primers were designed using CODEHOP (Rose et al. 1998).

ANT.txt. A text file of the alignment of ANT-related sequences in nexus format used in phylogenetic analysis.

BEL1.txt. A text file of the alignment of BEL1-related sequences in nexus format used in phylogenetic analysis.

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