Annals of Botany **128**: 217–230, 2021
doi: 10.1093/aob/mcab059, available online at www.academic.oup.com/aob

ANNALS OF BOTANY
Founded 1887

Deciphering the evolution of the ovule genetic network through expression analyses in *Gnetum gnemon*

Cecilia Zumajo-Cardona[1,2,] and Barbara A. Ambrose[2,*]

[1]New York Botanical Garden, Bronx, NY 10458, USA and [2]The Graduate Center, City University of New York, New York, NY 10016, USA
**For correspondence. E-mail bambrose@nybg.org*

Received: 18 March 2021 Editorial decision: 29 April 2021 Accepted: 30 April 2021 Electronically published: 7 May 2021

• **Background and aims** The ovule is a synapomorphy of all seed plants (gymnosperms and angiosperms); however, there are some striking differences in ovules among the major seed plant lineages, such as the number of integuments or the orientation of the ovule. The genetics involved in ovule development have been well studied in the model species *Arabidopsis thaliana*, which has two integuments and anatropous orientation. This study is approached from what is known in arabidopsis, focusing on the expression patterns of homologues of four genes known to be key for the proper development of the integuments in arabidopsis: *AINTEGUMENTA* (*ANT*), *BELL1*, (*BEL1*), *KANADIs* (*KANs*) and *UNICORN* (*UCN*).
• **Methods** We used histology to describe the morphoanatomical development from ovules to seeds in *Gnetum gnemon*. We carried out spatiotemporal expression analyses in *G. gnemon*, a gymnosperm, which has a unique ovule morphology with an integument covering the nucellus, two additional envelopes where the outermost becomes fleshy as the seed matures, and an orthotropous orientation.
• **Key Results** Our anatomical and developmental descriptions provide a framework for expression analyses in the ovule of *G. gnemon*. Our expression results show that although *ANT*, *KAN* and *UCN* homologues are expressed in the inner integument, their spatiotemporal patterns differ from those found in angiosperms. Furthermore, all homologues studied here are expressed in the nucellus, revealing major differences in seed plants. Finally, no expression of the studied homologues was detected in the outer envelopes.
• **Conclusions** Altogether, these analyses provide significant comparative data that allows us to better understand the functional evolution of these gene lineages, providing a compelling framework for evolutionary and developmental studies of seeds. Our findings suggest that these genes were most likely recruited from the sporangium development network and became restricted to the integuments of angiosperm ovules.

Key words: *AINTEGUMENTA*, *BELL1*, Gnetales, integument, *KANADI*, seed evolution, neosynangial hypothesis, *UNICORN*.

INTRODUCTION

The seed is a synapomorphy of all extant seed plants (gymnosperms and angiosperms), and develops from an ovule or integumented megasporangium. As sporangia are conserved throughout vascular plants, the origin of the integument is a defining step in seed evolution (Brenner and Stevenson, 2006). There are some key differences in the number of integuments present in seed plants as well as accessory structures that cover seeds in gymnosperms. Due to extra structures covering the nucellus, the ovules of Gnetales (*Ephedra*, *Gnetum*, *Welwitschia*) are strikingly different from those of all other extant gymnosperms. In the genera *Ephedra* and *Welwitschia* two envelopes cover the nucellus, and in the genus *Gnetum* three envelopes cover it (Martens, 1971; Takaso, 1985; Takaso and Bouman, 1986; Endress, 1996; Rydin *et al.*, 2010). Each of the three envelopes in *Gnetum* has been variously interpreted as integuments, a cupule or a megasporophyll based on comparisons with pteridosperms (extinct seed-bearing fern-like plants), other gymnosperms or angiosperms (Martens, 1971; Takaso and Bouman, 1986). In all three genera of Gnetales, it is generally agreed that the inner envelope is homologous to an integument that elongates and forms the micropyle; the homology of the additional envelopes is still debated but it is not debated that these form a seed coat unique to Gnetales (Takaso and Bouman, 1986; Ickert-Bond, 2003; Hollander and Vander Wall, 2009; Hollander *et al.*, 2010; Ickert-Bond and Rydin, 2011). Furthermore, the relationships among seed plants (Ginkgoales, Gnetales, Cycadales, Coniferales and angiosperms) are still debated. With morphological data, Gnetales and angiosperms are sister clades, which is known as the 'anthophyte' hypothesis (Crane, 1985; Doyle and Donoghue, 1986, 1992; Loconte and Stevenson, 1990). This is due to extensive morphological convergences between Gnetales and angiosperms, which include the presence of an additional integumentary envelope (Crepet *et al.*, 1991; Crane *et al.*, 1995). On the other hand, molecular data have recovered different topologies according to the genes and analyses used, but recent analyses indicate that Gnetales are nested within Coniferales (Ruhfel *et al.*, 2014; Wickett *et al.*, 2014; Forest *et al.*, 2018).

Focusing specifically on the differences between Gnetales and angiosperms, it is worth noting that while in Gnetales the initiation sequence of the envelopes is acropetal (i.e. the outer envelope develops first and then the inner envelope), in most angiosperms it is basipetal, i.e. the integument (inner envelope) develops first (Takaso, 1985; Endress, 1996; Rydin *et al.*, 2010). On the other hand, regarding the development of the envelopes in Gnetales, the outer envelope emerges in a horseshoe shape, starting on two sides of the ovule and later becoming uniformly distributed around the entire ovule, which is known as bilobed development (Takaso, 1984, 1985). Finally, in Gnetales the integument is vascularized with four bundles (Takaso, 1985; Rydin *et al.*, 2010) while in most angiosperms the integuments are not vascularized (Takaso, 1985; Endress, 2010, 2011; Rydin *et al.*, 2010).

A number of functional studies, mainly focused on the model species *Arabidopsis thaliana* (arabidopsis; reviewed in Colombo *et al.*, 2008), have revealed that the initiation, identity and development of the integuments involve a complex molecular circuitry that includes several transcription factors (Elliott *et al.*, 1996; Baker *et al.*, 1997; Skinner *et al.*, 2004). For the development of the ovule, in arabidopsis *WUSCHEL* (*WUS*) is a key gene; it is expressed in the nucellus and generates a downstream signal for integument development (Gross-Hardt *et al.*, 2002). In gymnosperms, the *WUS* homologue *GgWUS*/*WOX5* is expressed in the nucellus and the pollen cones of *Gnetum gnemon* (Nardmann *et al.*, 2009). Initially in arabidopsis, the MADS-box genes *SEEDSTICK* (*STK*), *SHATTERPROOF1* (*SHP1*) and *SHP2* act redundantly to determine the initiation of integument primordia (Favaro *et al.*, 2003; Pinyopich *et al.*, 2003; Brambilla *et al.*, 2007; Losa *et al.*, 2010), but the expression patterns of the homologue, *GGM3*, in *Gnetum* are different: it is expressed in the nucellus, integument and the pollen cones (Becker *et al.*, 2003).

Later, in the development of the ovule, the initiation of the integument also seems to be determined by *AINTEGUMENTA* (*ANT*), the main role of which is cell proliferation, affecting the initiation and proper growth of plant organs. In ovules of *ant* mutants there are no integuments as integument primordia are not formed and megasporogenesis is blocked (Elliott *et al.*, 1996; Klucher *et al.*, 1996; Baker *et al.*, 1997; Gasser *et al.*, 1998). *BELL1* (*BEL1*) is similar to *ANT*: *bel1* mutants do not develop integuments; however, unlike *ant*, *bel1* shows significant growth in the chalazal region, where an amorphous structure develops instead of integuments (Robinson-Beers *et al.*, 1992; Modrusan *et al.*, 1994; Ray *et al.*, 1994; Reiser *et al.*, 1995; Brambilla *et al.*, 2007; Bencivenga *et al.*, 2012).

Once the integuments are initiated, the planar development of the integument is controlled by another set of genes, members of the large transcription factor families *KANADI* (*KAN*) and *Class III HD-ZIP* (*C3HDZ*), which are known to determine the polarity of the lateral organs, by regulating a common set of direct target genes, many of which are linked to auxin signalling (Kerstetter *et al.*, 2001; Izhaki and Bowman, 2007; Reinhart *et al.*, 2013; Huang *et al.*, 2014). In arabidopsis, there are four *KANADI* (*KAN*) paralogues, *KAN1* to *4* (McAbee *et al.*, 2006), among which *KAN1* and *KAN2* also play a role in the development of the outer integument, determining its abaxial polarity (Kerstetter *et al.*, 2001; Bowman *et al.*, 2002; Eshed *et al.*, 2001, 2004; McAbee *et al.*, 2006). *ABERRANT TESTA SHAPE* (*ATS*) also known as *KANADI 4*, determines the abaxial polarity of the inner integument, resulting in a mutant phenotype that shows the two integuments fused (Leon-Kloosterziel *et al.*, 1994; McAbee *et al.*, 2006; Kelley *et al.*, 2012). *UNICORN* (*UCN*) is another gene that plays a role in the planar development of the integuments and is expressed in the outermost cell layers of the outer integument. The *ucn* mutants exhibit multicellular protrusions, also described as extra-integuments that develop from the outer integument (Schneitz *et al.*, 1997; Enugutti *et al.*, 2012, 2013).

Studies on the molecular genetics of ovule development have mainly focused on arabidopsis and a few other angiosperms (Dong *et al.*, 2000; Yamada *et al.*, 2003; Brown *et al.*, 2010; Dash and Malladi, 2012; Lora *et al.*, 2015; Skinner *et al.*, 2016; Arnault *et al.*, 2018). Expression studies of genes involved in the polarity of the integument in the early diverging angiosperm *Amborella trichopoda* suggest that this genetic network is conserved across angiosperms (Arnault *et al.*, 2018). Although these studies in gymnosperms are still rare, in *Gnetum parvifolium* and *Pinus thunbergii* the expression of *ANT* homologues, *GpANTL1* and *PtANTL1*, respectively, has been studied in the young strobili, where they were found to be expressed in the nucellus and primordia of all envelopes (Yamada *et al.*, 2008). The study of these genes in gymnosperms is of great importance in improving our understanding of the evolution and development of the ovule. Spatiotemporal expression analyses constitute a solid tool to assess their putative function, given the lack of functional genetic tools for these taxa.

Our study focused on *G. gnemon*, which has a key phylogenetic position and apparent morphological similarities to angiosperms, and is fundamental for assessing the evolution of these genes in seed plants. Determining if their role in the development of the integument is conserved outside angiosperms allows us to better understand their impact on the morphological evolution of the ovules and to elucidate whether the ovule genetic network is conserved in seed plants. Moreover, an insight into the genes acting in integument development makes it possible to clarify the nature of the envelopes in *G. gnemon* from a molecular perspective. We have performed detailed spatiotemporal expression analyses for six homologues of the four gene families *ANT*, *BEL1*, *KAN* and *UCN*, which have been previously identified with phylogenetic analyses and named *GneANT*, *Melbel1*, *GnmoKAN1* and *2*, and *GnmoUCN* and *GnmoUCN2* (Becker *et al.*, 2002; Kim *et al.*, 2006; Zumajo-Cardona and Ambrose, 2020).

These expression analyses made it possible to establish that the genes initially described as being involved in the development of the integument in angiosperms are not conserved in all seed plants. In turn, these genes appear to be involved in the development of the megasporangium in *G. gnemon*, suggesting that the ancestral function of the genes may be the development of sporangia. Furthermore, according to the expression patterns reported here in early-divergent seed plants, our results provide evidence that supports the interpretation of integuments as sterilized sporangia. Given the complexity of the ovule genetic network, it is difficult for us to extrapolate the results obtained here to other gymnosperms, and studies in representatives of Ginkgoales, Cycadales and Coniferales are still required.

MATERIALS AND METHODS

Anatomy of the strobili and ovules of Gnetum gnemon

Strobili at different developmental stages were collected from the Nolen Greenhouses at the New York Botanical Garden (Voucher 2153/2002*C; NYBG) and immediately fixed in formaldehyde–acetic acid–ethanol (FAA; 3.7 % formaldehyde, 5 % glacial acetic acid, 50 % ethanol) for 3 h. The fixed material was manually dehydrated through an alcohol–Histo-Clear II (National Diagnostics, Atlanta, GA) series and embedded in Paraplast X-TRA (Fisher Healthcare, Houston, TX, USA). The samples were sectioned at 8–12 µm with an AO Spencer 820 (GMI, MN, USA) rotary microtome. Sections were stained with Johansen's safranin, to identify lignification, cuticle and accumulation of tannins, and 0.5 % Astra Blue and mounted in Permount (Fisher Scientific, Pittsburgh, PA, USA). Sections were viewed with a Zeiss Axioplan compound microscope equipped with a Nikon DXM1200C digital camera with ACT-1 software.

Expression analyses by in situ *hybridization*

Gnetum gnemon strobili were collected as described in the preceding section for the anatomical study. After a 3-h fixation, samples were dehydrated in an ethanol series and then transferred to fresh Paraplast X-TRA and stored at 4 °C until use. Samples were sectioned on a Microm HM3555 rotary microtome at 10 µm. Sequences were initially identified through a BLAST search using arabidopsis sequences as query (*BEL1* = At5g41410; *KAN1* = At5g16560; *KAN2* = At1g32240; *KAN3* = At4g17695; *KAN4* = At5g42630; *UCN* = At1g51170). The homology of these sequences has been established with previous phylogenetic analyses by several authors (*BELL1*, named *Melbel1*, initially characterized by Becker *et al.*, 2002; *ANT* by Yamada *et al.*, 2008; *Melbel1*, *KANs* and *UCNs* by Zumajo-Cardona and Ambrose, 2020). Maximum likelihood analyses with selected sequences were performed using the RaxML-BlackBox available through the Cipres Portal (Stamatakis *et al.*, 2008; Miller *et al.*, 2012), to corroborate the homology of the *Gnetum* sequences used here (Supplementary Data Figs S1 and S2). DNA templates for RNA probe synthesis were obtained by PCR amplification of 300- to 500-bp fragments. To ensure specificity, the probe templates were designed to amplify the flanks of the domains: the *Melbel1* probe amplifies the 5′ sequence flanking the homeodomain; *GneKAN1* amplifies the 3′ region flanking the GARP domain; the *GneKAN2* sequence is incomplete but the probe was designed towards the 3′ end as well; *GneUCN1* and *2* probes amplify a region at the 3′ end with no functional motifs (Supplementary Data Table S1, Fig. S3). Fragments were cleaned using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). Digoxigenin-labelled RNA probes were prepared using T7 RNA polymerase (Roche, Switzerland), murine RNAse inhibitor (New England Biolabs, Ipswich, MA, USA) and Digoxigenin d-UTP RNA Labeling Mix (Roche, Switzerland) according to each manufacturer's protocol. RNA *in situ* hybridization was performed according to Ambrose *et al.* (2000) and Ferrandiz *et al.* (1999), optimized to hybridize overnight at 55 °C. *In situ* hybridized sections were subsequently dehydrated and permanently mounted in Permount (Fisher Scientific, Pittsburgh, PA, USA). Sections were viewed and photographed with a Zeiss Axioplan compound microscope equipped with a Nikon DXM1200C digital camera.

RESULTS

Anatomy of the ovules and seeds of G. gnemon

Ovules and microsporangia form on specialized structures called strobili. Strobili develop in the axil of a leaf; each strobilus consists of a long axis bearing numerous pairs of decussate bracts. Some strobili are bisexual; both ovules and microsporangia develop in the axil of the same bract, all around the axis (Chamberlain, 1935), thus forming at each node of the strobilus an upper ring of ovules and several rings of basipetal microsporangia (Fig. 1A). However, in *G. gnemon* some strobili are only ovulate (Fig. 1A–D; Takaso and Bouman, 1986). The ovules are characterized by an integument and two additional envelopes, resulting in three protective layers covering the megagametophyte. As the ovule matures the outermost layer changes colour from green and coriaceous (Fig. 1B) to yellow, indicating that it is ready to be pollinated (Fig. 1C). After pollination, it turns into a red fleshy seed (Fig. 1D). The layers covering the megagametophyte, going from the inside to the outside, are the integument, the middle envelope and the outer envelope (Endress, 1996; Rydin *et al.*, 2006; Fig. 1E). The microsporangia are unilocular (Fig. 1F). The ovules that develop on a staminate strobilus (forming a bisexual strobilus) have one envelope, and hence these ovules have an integument covered by an additional envelope (Fig. 1F), whereas the ovules that develop on an ovulate strobilus have three layers covering the megagametophyte, as shown by the well-developed ovule illustrated in Fig. 1G (Beccari, 1877; Strasburger, 1879; Takaso and Bouman, 1986). A well-developed ovule has the envelopes and the integument completely developed and overtopping the nucellus. At the following stage, the megaspore mother cell is formed in the centre of the nucellus (Fig. 1H). After pollination, the nucellus begins to degenerate and the integument closes over the nucellus. The three envelopes form the seed coat: the outer envelope starts to become fleshy, leaving apparent spaces between the cells forming the 'fleshy outer' region of the seed; the middle envelope is sclerenchymatic, forming the 'stony layer' and the inner envelope (integument) compresses, forming a 'papery layer' (Takaso and Bouman, 1986; Fig. 1I). All layers surrounding the megagametophyte are completely separated from each other, including the integument (the inner layer), which is also separated from the megagametophyte (Fig. 1F–I). The ovules born on bisexual strobili are usually abortive; if the ovule is young it does not affect the development of microsporangia on the same node (Fig. 1F). However, if the ovule has reached an advanced stage of development, nearby microsporangia will abort while those that are further from the mature ovule will develop properly (Fig. 1H; Chamberlain, 1935).

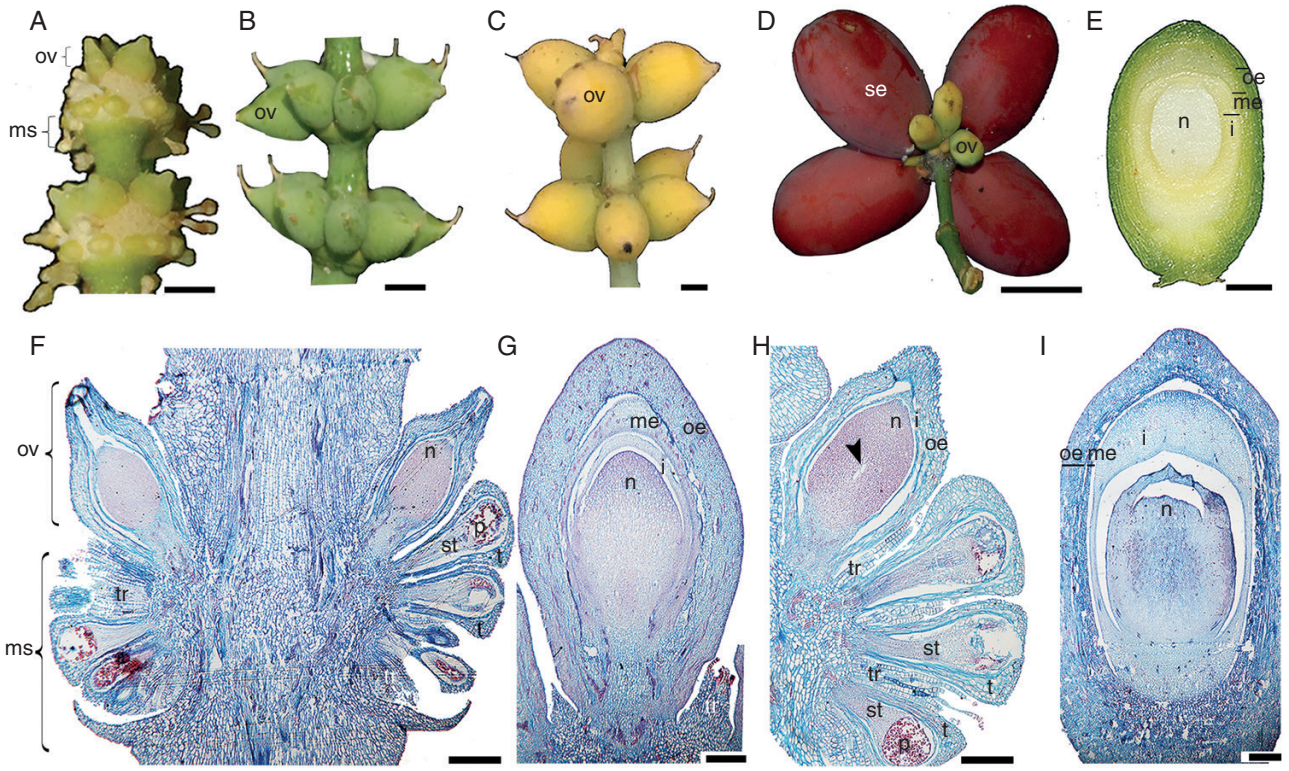

FIG. 1. Morphoanatomical development of the ovules in *G. gnemon*. (A) Strobili with a ring of ovules and rings of microsporangia developing basipetally at each node. (B, C) Ovules at different stages of development, developing on ovulate strobili. (D) Red fleshy seed. (E) Inside of the seed showing three different layers protecting the nucellus. (F) longitudinal section throughout strobili, sterile ovule with one envelope surrounding the integument and the oldest microsporangium next to the ovule. (G) Ovule with the nucellus covered by the integument, middle envelope and outer envelope. (H) Strobilus with functional ovule, where the gametophyte has reached the free nuclear stage; the functional microsporangium is at the bottom. (I) Mature ovule after fertilization. The middle envelope is fusing with the outer envelope and the nucellus has started to degenerate. i, integument; me, middle envelope; ms, microsporangia; n, nucellus; oe, outer envelope; ov, ovule; p, pollen; se, seed; st, sporogenous tissue; t, tapetum; tr, trichome. Scale bars: (A–C, E) = 0.5 cm, (D) = 1 cm, (F–H) = 50 µm, (I) = 25 µm.

Expression of AINTEGUMENTA *homologue*

Expression analyses have been previously performed during very early stages of ovule development in *Gnetum parviflorum* (*GpANTL1*), where expression was detected in the abaxial side of all envelope primordia, integument primordia and the nucellar tip (Yamada *et al.*, 2008). Here we investigated the expression of one *ANT* homologue in *Gnetum* that has been previously identified with phylogenetic analyses (Yamada *et al.*, 2008; Supplementary Data Fig. S2). Due to the availability of plant material, our expression analyses focused on strobili later in development. During early stages of ovule development *GneANT* is expressed in the nucellus and the entire integument; there is no expression in the outer envelope (Fig. 2A). As the micropyle develops and the ovule is ready to be fertilized, expression in the integument is restricted to the apical region that forms the micropyle (Fig. 2B). *GneANT* expression is maintained in the nucellus and the integument throughout ovule development, including the stage when the megaspore mother cell is formed (Fig. 2C) and after meiosis (Fig. 2D). *GneANT* expression is also detected in the sporogenous tissue of the microsporangia and the pollen grains (Fig. 2D).

Expression of BELL1 *homologue*

A homologue of *BELL1* in *G. gnemon*, named *Melbel1*, has been identified by phylogenetic analyses (Becker *et al.*, 2002). Thus, to better understand the putative function of *BEL1* homologues in ovule development of *G. gnemon*, we performed spatiotemporal expression analyses at different stages of development. In young sterile developing ovules, *Melbel1* is detected at low levels in the nucellus and pollen grains (Fig. 2E). In the next stage, as the nucellus continues growing, these expression patterns are maintained (Fig. 2F). As the ovule matures, *Melbel1* is still expressed in the nucellus and in the megaspores (after meiosis). Strikingly, no expression is detected in the apical region of the nucellus, the pollen chamber or the integument or the outer envelope (Fig. 2G).

Expression of KANADI *homologues*

Two *KAN* homologues have been identified for *G. gnemon*, named *GnmoKAN1* and *GnmoKAN2* (Zumajo-Cardona and Ambrose, 2020). The position of these two sequences within gymnosperms is not yet clear, but all gymnosperm homologues are sister clades to the angiosperm-specific clades *KAN1* and *KAN2/3*, which are also involved in integument polarity in arabidopsis (Kerstetter *et al.*, 2001; Bowman *et al.*, 2002; Eshed *et al.*, 2004; McAbee *et al.*, 2006; Zumajo-Cardona and Ambrose, 2020). With the spatiotemporal expression analyses we detected that the two *G. gnemon* homologues show different expression patterns, suggesting some degree of sub-functionalization (Fig. 3). *GnmoKAN1* is expressed in the nucellus and in the apical portion

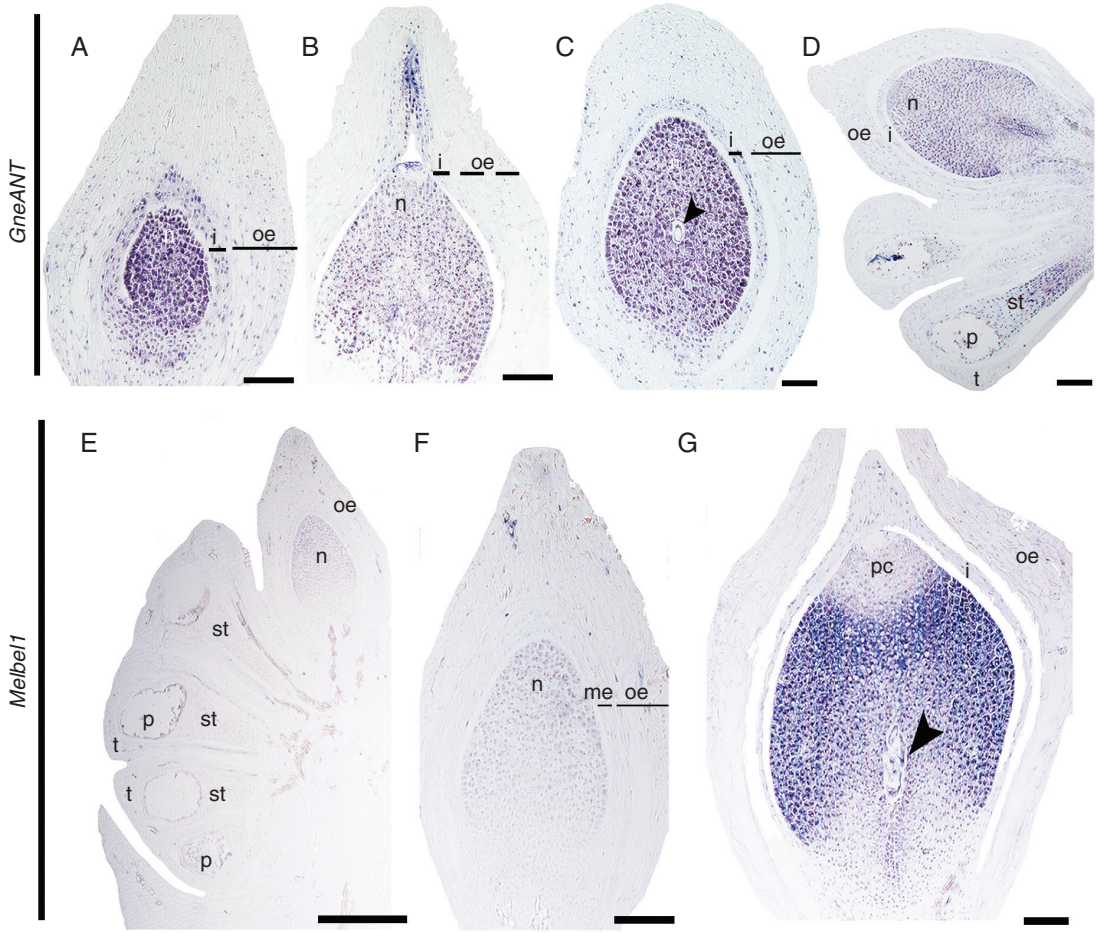

Fig. 2. Expression analyses at different stages of ovule development using *in situ* hybridization. (A–D) Expression patterns of *GneANT*. (E–G) Expression of *Melbel1*. Arrowhead points to the megaspore; i, integument; me, middle envelope; n, nucellus; oe, outer envelope; ov, ovule; p, pollen; st, sporogenous tissue; t, tapetum. Scale bars: (A–C, G) = 25 µm, (D, F) = 50 µm, (E) = 100 µm.

of the integument during early stages of ovule development (Fig. 3A), and its expression patterns are maintained throughout ovule development, when the micropyle is formed (Fig. 3B), and as the ovule matures, when it is also found to be expressed in the megaspore mother cell (Fig. 3C). However, *GnmoKAN2* is expressed at low levels in the nucellus of a young developing ovule (Fig. 3D). At the next stage, when the megaspore begins to develop, *GnmoKAN2* is specifically expressed in the megaspore mother cell and towards the apical region of the integument (Fig. 3E). These expression patterns are maintained as the megaspore undergoes meiosis (Fig. 3F).

Expression of UNICORN *homologues*

Two *UCN* homologues have been identified in *G. gnemon*, named *GnmoUCN* and *GnmoUCN2*, both belonging to the same clade within gymnosperms (Zumajo-Cardona and Ambrose, 2020). However, the two paralogues show different expression patterns (Fig. 4). *GnmoUCN* is expressed in the nucellus in the young developing ovule (Fig. 4A). The expression in the nucellus is maintained as the ovule develops and forms the megaspore, where it is also expressed. In addition, *GnmoUCN* expression is also found in the apical region of the integument forming the micropyle (Fig. 4B). Low levels of *GnmoUCN* expression are detected in the sporogenous tissue of the microsporangia (Fig. 4C). On the other hand, *GnmoUCN2* is expressed specifically in the megaspore mother cell (Fig. 4D) and this expression is maintained as the megaspore undergoes meiosis. At this stage, *GnmoUCN2* is also expressed in the apical region of the integument (Fig. 4E). These expression patterns are maintained when the micropyle begins to close; moreover, *GnmoUCN2* expression is also detected in the pollen chamber, in the apical region of the nucellus, as it begins to degenerate (Fig. 4F). No expression is detected in the microsporangium (Fig. 4G).

DISCUSSION

Within the ovule, the integument is one of the most important structures, with roles spanning from the protection of the female gametophyte to reproduction. In angiosperms, cross-talk between the integuments with the megagametophyte and with the fertilization products ensures the proper development of the seed (Figueiredo and Köhler, 2016). When the integuments become the seed coat, the cross-signalling with the endosperm

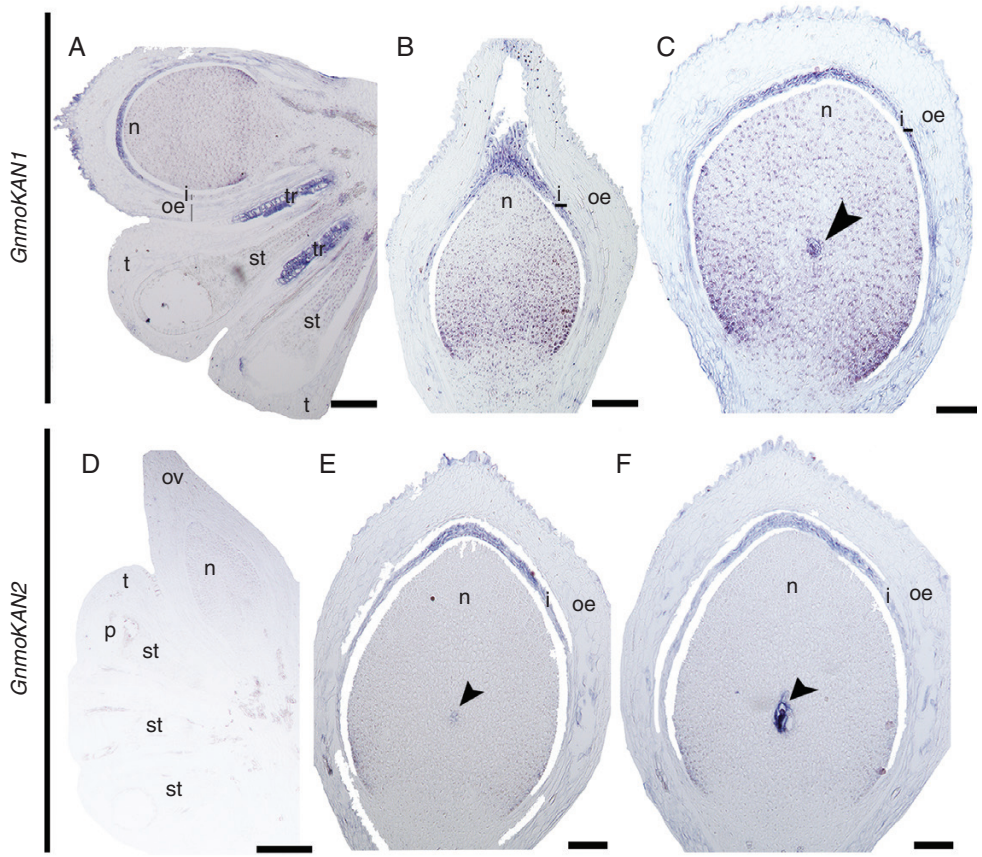

FIG. 3. Expression analyses of the two *KAN* homologues in *G. gnemon* at different stages of ovule development. (A–C) Expression patterns of *GneKAN1*. (D–F) Expression patterns of *GneKAN2*. Arrowhead points to the megaspore; i, integument; me, middle envelope; n, nucellus; oe, outer envelope; ov, ovule; p, pollen; st, sporogenous tissue; t, tapetum. Scale bars: (A) = 100 µm, (B, F) = 25 µm, (C–E) = 50 µm.

ensures its coordinated growth, and in addition the seed coat ensures successful seed dispersal, protects the embryo from stress and influences dormancy, germination and seed longevity (Figueiredo and Köhler, 2014; Neuman and Hay, 2020).

The ovule is a salient synapomorphy of all seed plants, but there are major morphological differences across seed plants. Regarding *Gnetum*, its ovules have a unique morphology not found in any other plant. With an orthotropous orientation, its ovules have an integument, which is separated from the nucellus, and two additional envelopes that cover the ovule; the seed is fleshy, thus ensuring seed dispersal by animals (Fig. 1D; Takaso and Bouman, 1986). In contrast, the ovules of arabidopsis exhibit the typical ovule morphology of angiosperms, with two integuments completely fused to the nucellus and with anatropous orientation due to the asymmetrical growth of the outer integument (Schneitz *et al.*, 1995). In gymnosperms, the integument plays key roles in fertilization, as the ovule directly receives the pollen. In Gnetales, the integuments elongate over the nucellus, leaving a small opening, the micropyle, producing a pollination drop on which the pollen lands; subsequently the pollination drop is withdrawn to the pollen chamber, on which the pollen germinates; at this stage the micropyle closes (Takaso and Bouman, 1986; Tomlinson *et al.*, 1991; reviewed in Rudall, 2021).

Understanding the functional evolution of the genetic network of ovule development and its impact on the morphological evolution of land plants remains difficult because it has been mainly studied in arabidopsis (Colombo *et al.*, 2008), including *ANT*, *BEL1*, *KAN*s and *UCN* genes, involved in integument development. Each of these genes belongs to a different family of transcription factors, each with a unique evolutionary history and having, in turn, multiple independent duplication events across seed plants and with homologues within land plants (Fig. 5; Kim *et al.*, 2006; Zumajo-Cardona and Ambrose, 2020).

AINTEGUMENTA, *from sporangia development in early embryophytes to integument identity in seed plants*

ANT is a plant-specific gene, a member of the large transcription factor family *APETALA2/ETHYLENE-RESPONSIVE ELEMENT-BINDING FACTOR* (*AP2/ERF*; Nole-Wilson and Krizek, 2006; Kim *et al.*, 2006). The *ANT* homologue in the fern *Ceratopteris richardii*, *CerANT*, is expressed in the sperm, in the archegonial neck canal just before fertilization (gametophyte structure) and in the fertilized egg, the zygote, but not in the egg cell before fertilization; expression is also detected in the fiddlehead (sporophyte; Bui *et al.*, 2017). Overexpression of *CerANT* promotes apogamy, where the sporophyte develops from the gametophyte but without fertilization of the gametes (Bui *et al.*, 2017). Expression analyses in young developing ovules in the gymnosperms *P. thunbergii*

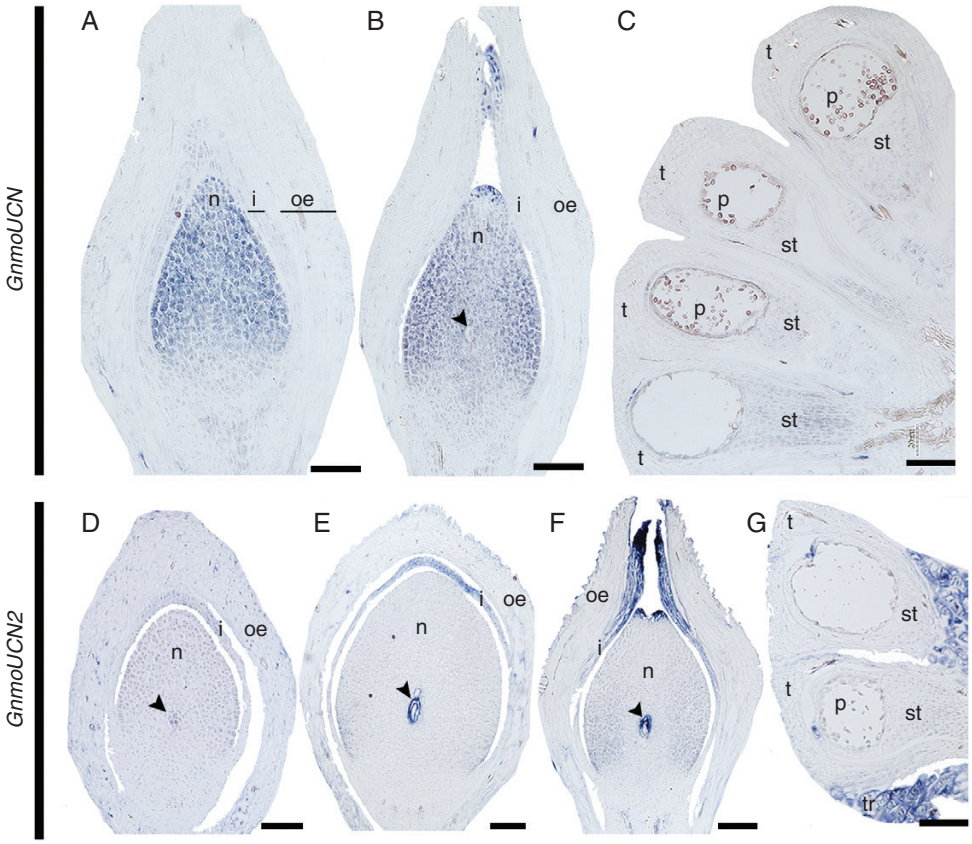

Fig. 4. Expression analyses of the two *UCN* homologues in *G. gnemon* at different stages of ovule development. (A–C) Expression patterns of *GneUCN*. (D–G) Expression patterns of *GneUCN2*. Arrowhead points to the megaspore; i, integument; me, middle envelope; n, nucellus; oe, outer envelope; ov, ovule; p, pollen; st, sporogenous tissue; t, tapetum. Scale bars: (A–D, G) = 50 µm, (E–F) = 25 µm.

(*PtANTL1*) and *G. parvifolium* (*GpANTL1*) show expression in the nucellus and integument (Yamada *et al.*, 2008). Similarly, we found that the expression in the nucellus and in the integument is maintained throughout ovule development in *G. gnemon*. These results suggest that the expression of *ANT* homologues is conserved in *P. thunbergii* and *Gnetum* spp. and that it is maintained throughout ovule development (Yamada *et al.*, 2008; Fig. 2A–D). In addition, we detected expression in the pollen grains (i.e. microspores), which suggests that *ANT* homologues were retained in gymnosperms as key factors in the development of the megaspores and microspores (i.e. gametophyte development), similar to what is found in ferns (Fig. 2A–D).

In angiosperms, in species such as *Petunia* × *hybrida*, *Antirrhinum majus* (Manchado-Rojo *et al.*, 2014) and *Malus domestica* (Dash and Malladi, 2012), *ANT* homologues have been identified and functionally characterized, showing that these genes act in the development of both integuments, or the only integument in *Petunia*, as well as in the control of leaf size (Dash and Malladi, 2012; Manchado-Rojo *et al.*, 2014). Likewise, in arabidopsis the *ant* mutants do not develop integuments, megasporogenesis is blocked, the number of floral organs decreases, and the size of the leaves is smaller (Elliott *et al.*, 1996; Klucher *et al.*, 1996; Baker *et al.*, 1997; Gasser *et al.*, 1998). Studies in arabidopsis showed that pleiotropic roles of *ANT* in plant development are the result of its control over cell proliferation during organogenesis, affecting initiation, growth and intrinsic organ size, including ovules and floral organs as well as shoots and leaves (Elliott *et al.*, 1996; Mizukami and Fischer, 2000), which seems to be the conserved function across core eudicots.

Furthermore, it appears that expression patterns of *ANT* are not conserved in seed plants; in arabidopsis expression is restricted to the integuments, but in *Gnetum* it is found not only in the integuments but also in the nucellus (Figs 2 and 5C). It is worth noting that *ANT* is expressed in the micropyle region, where the integument has active cell division (Fig. 2C), and that it is not expressed in either of the outer envelopes, indicating that these structures use genes other than *ANT* for cell proliferation.

Altogether, the ancestral function of *ANT* seems to be in gametophytic development within the sporangia, as seen in ferns and in gymnosperms (Bui *et al.*, 2017; Yamada *et al.*, 2008; Fig. 2A–D). Apparently, during integument evolution in seed plants, *ANT* homologues have been recruited for its development (Yamada *et al.*, 2008). It is important to note that *ANT* in arabidopsis is a gene with pleiotropic roles, being involved not only in integument initiation but also in primordia development of all plant organs except roots (Elliott *et al.*, 1996).

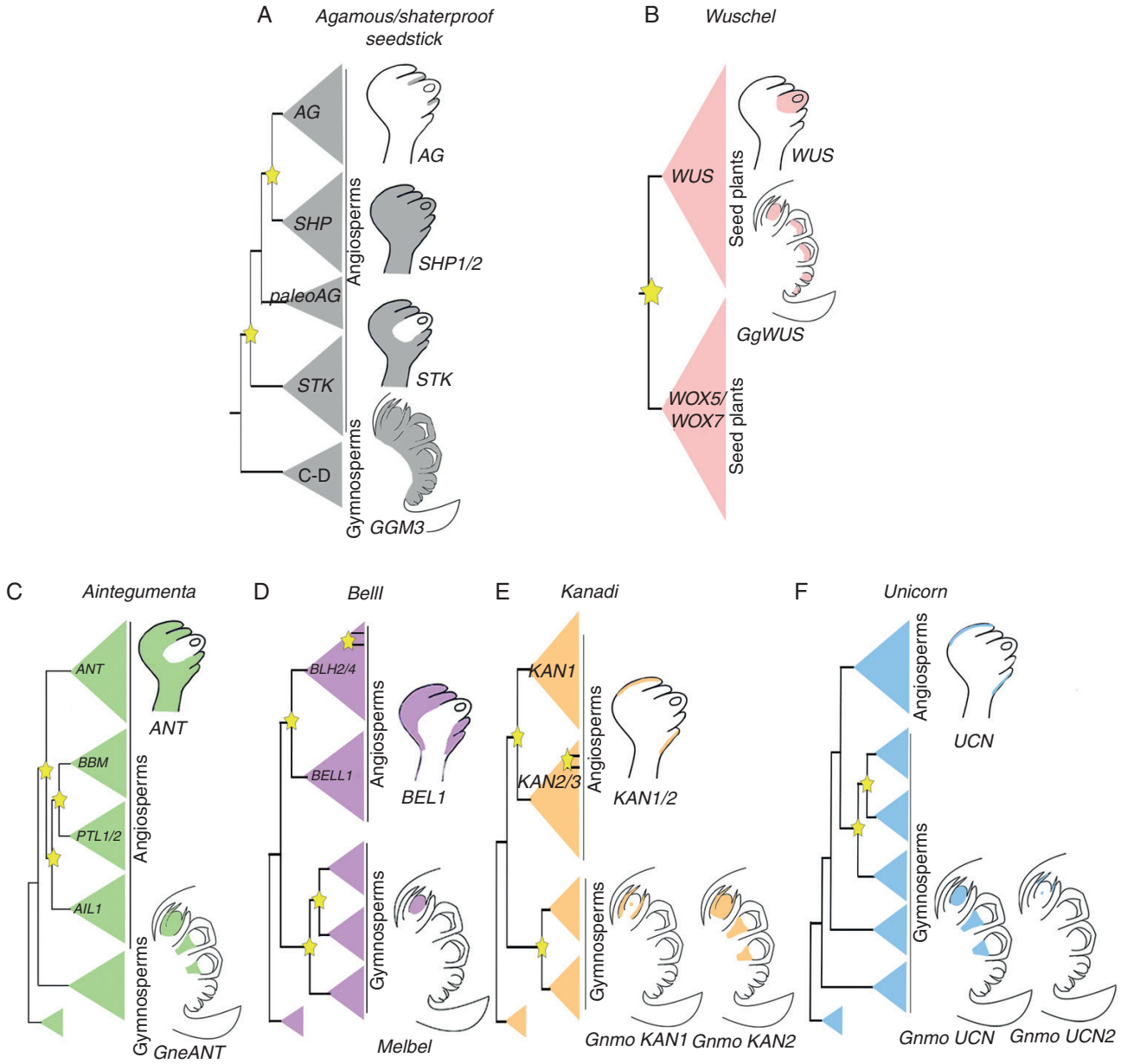

FIG. 5. Schematic representation showing the expression of the different genes known to be involved in ovule development in *A. thaliana* and what is known so far in *Gnetum* spp. Information on seedless plants (ferns, lycophytes or mosses) was included when available for reproductive structures . MADS-box gene data (A) are based on Ray *et al.* (1994), Becker *et al.* (2002), Losa *et al.* (2010) and Pabón-Mora *et al.*, 2014. *WUSCHEL* data (B) are based on Gross-Hardt *et al.* (2002) and Nardmann *et al.* (2009). Yellow stars pointing to duplication events in each gene lineage.

Changes in the expression patterns of BELL1 *suggest major changes in its interactions among land plants*

BEL1 belongs to the Three Amino acid Loop Extension (TALE) homeobox family of transcription factors that are conserved among eukaryotes (Reiser *et al.*, 1995; Bürglin, 1997; Bellaoui *et al.*, 2001). Functional studies in the moss *Physcomitrella patens* show that *PpBELL1* is a master regulator of the gametophyte–sporophyte transition; loss of function of *PpBELL1* generates bigger egg cells unable to form embryos, suggesting that *BELL1* was the key to facilitating the diversification of land plants (embryophytes; Horst *et al.*, 2016). Expression in the gymnosperm *G. gnemon*, where the homologue *Melbel1* is expressed in the nucellus and the megaspore after meiosis, suggests that the function in the proper formation of the egg cell may be conserved (Fig. 2E–G). In angiosperms the function in ovule development seems to be conserved. In the monocot *Hordeum*, two *BEL1* homologues have been identified, *JUBEL1* and *2* showing expression in the meristematic tissues and primordia of the ovule (Müller *et al.*, 2001). In other angiosperms, such as *Malus domestica*, analyses of the *BELL1* homologue, known as *MDH1*, suggest that its function in the ovule is conserved to that in arabidopsis, where *BEL1* acts in the proper development of the integuments (Dong *et al.*, 2000). In arabidopsis, the *bel1* mutant causes significant

growth in the chalazal region, producing an asymmetrical fleshy structure at the base of the nucellus, but no true integument is formed. Thus, the function of *BEL1* in the development of the integument seems to be due to the interaction with the carpel identity dimer AGAMOUS-SEPATALLATA3 (AG/SEP3) and to the repression of WUSCHEL (WUS) towards the nucellus (Brambilla *et al.*, 2007). Another interaction, which may be related to the function of *BEL1* in integument formation shown by expression analyses, is the repression of SPOROCYTELESS (SPL), a master regulator of nucellus-forming pathways upregulating PIN-FORMED 1 (PIN1) and WUS (Sieber *et al.*, 2004; Bencivenga *et al.*, 2012; Yamada *et al.*, 2019).

We infer that the function of *BEL1* homologues in development of the egg cell may be conserved in bryophytes and gymnosperms (Fig. 2E–G; Horst *et al.*, 2016). Although our results do not cover embryo development, the expression patterns of *Melbel1* found in the nucellus and the megaspore mother cell suggest that it could be directly or indirectly involved in embryo development in *G. gnemon* as well. However, this function does not seem to be conserved in angiosperms, where *BELL1* homologues play key roles in formation of the ovule primordia and integument, suggesting major differences across seed plants. Overall, this leads us to suggest that major changes in the functional evolution of the *BELL1* gene lineage occurred following a duplication event that took place before the diversification of angiosperms (Fig. 5D).

Integument polarity genes KANADI *and* UNICORN *show different expression patterns in seed plants*

The *KAN* genes, a subset of the large *GARP* family of transcription factors, are present in all eukaryotes and are characterized by the plant-specific *GARP* DNA-binding domain (Riechmann *et al.*, 2000; Hosoda *et al.*, 2002; Zhang *et al.*, 2009). Expression studies in the lycophyte *Selaginella moellendorffii* show that there are three *KAN*-specific homologues differentially expressed throughout sporangium development, participating in its initiation (*SmKAN1*, *2*) through sporocyte formation (*SmKAN3*; Zumajo-Cardona *et al.*, 2019). In the fern *Equisetum hyemale* the expression of *KAN* homologues was assessed in vegetative tissue, where it was found in leaf primordia and the abaxial side of each leaf (Zumajo-Cardona *et al.*, 2019).

The expression patterns we found in the gymnosperm *G. gnemon*, where the two paralogues are expressed in the nucellus (megasporangium) and in the megaspore, are similar to those in *S. moellendorffii* (Fig. 3). Our results show different expression patterns between the two *G. gnemon* paralogues, suggesting a partial sub-functionalization event. While *GnmoKAN1* is expressed in the nucellus, integument and megaspore, *GnmoKAN2* is expressed in the megaspore and the apical region of the integument (Fig. 3). Expression or functional studies in the reproductive structures of ferns are still required to better hypothesize whether this function is conserved in lycophytes, ferns and gymnosperms.

In angiosperms, the *KAN* genes are known for their role in establishing leaf polarity, specifically the abaxial side of the leaf, similar to that found in ferns (Zumajo-Cardona *et al.*, 2019). This function has been shown to be conserved in monocot homologues: *Milkweed pod1* in *Zea mays* (Candela *et al.*, 2008) and *SHALLOT-LIKE1* in *Oryza sativa* (Zhang *et al.*, 2009). In arabidopsis they are also responsible for specifying the abaxial identity of integuments. *KAN1* and *2* are responsible for the planar identity of the outer integument, while *ATS* (also known as *KAN4*) is responsible for the planar identity of the inner integument (Leon-Kloosterziel *et al.*, 1994; Kerstetter *et al.*, 2001; Bowman *et al.*, 2002; Eshed *et al.*, 2004; McAbee *et al.*, 2006; Kelley *et al.*, 2012).

While *KAN1/2* expression is detected in the outer integument of arabidopsis, we only detected expression of *GmnoKAN1* and *GmnoKAN2* in the single integument of *G. gnemon* and not in any of the outer envelopes. Therefore, the development of these structures does not require *KAN* function. As for integument polarity, no observations have been reported in other angiosperms, and no polar expression was detected here in the integument of *Gnetum* (Fig. 3). Hence, three scenarios are possible: (1) the function in integument polarity is not conserved across seed plants; (2) there are major changes in the regulatory network involved in ovule development in *Gnetum*; or (3) polar expression patterns of *KAN* are only present during early stages of development. Although the anatomy of the integument and the envelopes is homogeneous, it is important to emphasize that expression and functional studies are still needed, focusing on the development of the ovule in different species of seed plants. Previous studies in lycophytes together with our findings allow us to hypothesize that the ancestral function of the *KAN* genes is in the development of the sporangium and that it is conserved among lycophytes and gymnosperms.

UNICORN (*UCN*) encodes a functional AGC VIII kinase and acts in determining the planar identity of the outer integument (Schneitz *et al.*, 1997; Enugutti *et al.*, 2012, 2013). *UCN* also appears to be implicated in the planar growth of other plant organs, such as petals (Enugutti *et al.*, 2012). *UCN* suppresses ectopic growth of integuments through two independent processes: by attenuating the protein kinase *3-PHOSPHOINOSITIDE-DEPENDENT PROTEIN KINASE 1* (*PDK1*) in the cytoplasm, which is involved in the stress response, and on the other hand by promoting growth (Flynn *et al.*, 2000) and repressing ABERRANT TESTA SHAPE (ATS) in the nucleus (Scholz *et al.*, 2019). There are two copies in *G. gnemon*: *GnmoUCN* and *GnmoUCN2*; although our results may not be conclusive for determining whether these homologues are also involved in integument polarity, it seems that this function is not conserved in *G. gnemon* because (1) *GnmoUCN* is expressed only in the nucellus (Fig. 4A-C), (2) *GnmoUCN2* is expressed in the megaspore and in the apical region of the integument that forms the micropyle (Fig. 4D–G), and (3) we found no differences in the expression of *UCN* homologues in *G. gnemon* between the adaxial and abaxial sides of the integument (Fig. 4).

In arabidopsis UCN is known to interact with ATS in planar development, an interaction that seems to be maintained with other KAN genes (Enugutti *et al.*, 2012; Enugutti and Shneitz, 2013). Interestingly, *GnmoKAN1* and *2* and *GnmoUCN2* are expressed in the distal region of the integument, which will form the micropyle; to assess if the interaction is maintained in *Gnetum* and their role in proximo-distal development of the integument, further studies are required.

Phylogenetic analyses show a complex evolutionary history for the *KAN* and *UCN* lineages (Zumajo-Cardona and Ambrose, 2020). Indeed, the lack of functional characterization outside arabidopsis does not allow us to predict the evolutionary history of this lineage. However, their function in integument polarity does not seem to be conserved between arabidopsis and *G. gnemon*. In gymnosperms, the *KAN* homologues predate a duplication event which resulted in *KAN1* and *KAN2/3* clades in angiosperms (Fig. 5E). This suggests that the *KAN* function in integument polarity may have occurred as a neofunctionalization event in angiosperms. Regarding the *UCN* lineage, it has undergone five gymnosperm-specific duplication events (Fig. 5F). While the angiosperm inner integument is considered to be homologous to the single gymnosperm integument (Crane, 1985; Doyle and Donoghue, 1986; Gasser and Skinner, 2019), interestingly, KAN and UCN homologues that are expressed in the arabidopsis outer integument are expressed in the single gymnosperm integument.

Evolution of the ovule development genes and what it may imply in the morphological evolution of ovules

The genes that we studied here are known to be involved in proper development of the integument (Baker *et al.*, 1997; Gasser and Skinner, 2019). However, for the integuments to begin to develop, the identity of the ovule must have been established. To gain a better understanding of the putative molecular evolution of the ovule genetic network, our discussion here focuses on what is known about these genes across seed plants. The ovule identity protein complex is formed by three MADS-box proteins: SEEDSTICK (STK), SEPALLATA (SEP) and SHATERPROOF (SHP), which stabilize the BEL1-SEP3-AGAMOUS (AG) complex, to regulate the identity of the integument (Colombo *et al.*, 2008). In addition, STK, SHP1 and SHP2 specify the fate of the integument cells, and later on ANT promotes the initiation and growth of these cells (Losa *et al.*, 2010). The expression patterns of *AG*, *SHP* and *STK* in arabidopsis are found in the placenta and expression is maintained in the ovule, where their expression diverges, becoming specific to different regions (Fig. 5A; Ray *et al.*, 1994; Losa *et al.*, 2010). Expression studies carried out on the homologue in *G. gnemon*, *GGM3*, revealed that it is expressed in the entire ovule and in the pollen cones (Fig. 5A; Becker *et al.*, 2002). These expression patterns as well as the evolution of the gene lineage suggest that these genes play pleiotropic roles in *Gnetum* and that, after multiple angiosperm-specific duplication events, these genes have become restricted to the ovule (Fig. 5A). Furthermore, the expression of *AG*, *SHP* and *STK* in the ovule further suggests a sub-functionalization event (Becker *et al.*, 2002; Kramer *et al.*, 2004; Pabón-Mora *et al.*, 2014).

In arabidopsis, *WUSCHEL* is well known for its function in meristem identity (Laux *et al.*, 1996; Mayer *et al.*, 1998; Schoof *et al.*, 2000). But in the ovule *WUS* is required for the proper establishment of the chalaza, the distal region of the ovule from which the integuments develop, and to induce the formation of the integuments. In fact, *wus* mutants do not develop integuments (Gross-Hardt *et al.*, 2002; Sieber *et al.*, 2004). Moreover, the expression of *WUS* is restricted to the nucellus, activating a downstream signal that derives from the nucellus and induces organ initiation in the adjacent chalaza cells (Fig. 5B), revealing that *WUS* activity is not in the cells where it is expressed, but forms a short-range signalling module repeatedly during plant development (Gross-Hardt *et al.*, 2002; Colombo *et al.*, 2008). Expression studies in the *Gnetum* homologue, known as *GgWUS*, exhibit expression in the nucellus, similar to that in arabidopsis ovules, suggesting that the expression and putative function of *WUS* in the ovule is conserved across seed plants (Fig. 5B; Nardmann *et al.*, 2009). The *WUS* clade appears to be seed-plant-specific as the result of multiple duplication events specific to this lineage, in the clade T3WOX where it belongs (Gehring *et al.*, 1990; Nardmann *et al.*, 2009; Wu *et al.*, 2019). In the fern *Ceratopteris richardii*, T3WOX homologues are expressed in the young tissues of the plant and in the root apical meristem (Nardmann and Werr, 2012; Youngstrom *et al.*, 2019). Due to the four major duplication events of this gene lineage and the differences in the expression patterns, the evolution of T3WOX genes is complex, with functional changes in the major plant lineages.

Class III HD-Zip genes in arabidopsis, including the paralogues *CORONA* (*CNA*), *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*), have also been reported to be involved in the proper establishment of the planar polarity of the integuments, where they are expressed adaxially (Sieber *et al.*, 2004; Kelley and Gasser, 2009; Kelley *et al.*, 2009). In addition, functional studies revealed that *BEL1*, *CNA*, *PHB* and *PHV* genes restrict *WUS* expression to the nucellus, probably independently (Yamada *et al.*, 2016). Homologues of *Class III HD-Zip* genes have been identified across land plants, vascular plants seem to have undergone independent duplication events and lycophyte homologues are preduplication genes (Vasco *et al.*, 2016). So far, no expression analyses have been performed in gymnosperms. But expression patterns in ferns show these genes on the adaxial side of the leaf. In addition, *Class III HD-Zip* homologues are expressed in the sporangia of the lycophyte *Selaginella moellendorffii* and the fern *Psilotum nudum*, suggesting that sporangia development may be the ancestral function of this gene lineage (Vasco *et al.*, 2016).

Our results allow us to conclude that the ancestral function of integument genes is most likely in sporangium development and that these genes were subsequently recruited for integument development in angiosperms (Fig. 5C–F). The *ANT*, *KAN* and *UCN* homologues were also found to be expressed in the integument of *G. gnemon.* However, in arabidopsis this expression seems to be restricted to the integuments. The evolutionary history of these genes has been shown to be complex and most of the gymnosperm homologues are pre-duplication genes (i.e. *BEL1*, *KAN* and *UCN*; Fig. 5). The major differences observed in the expression patterns, suggest that their specific functions in integument development may be the result of sub- or neo-functionalization events in angiosperms. However, expression studies are still required, at early stages of ovule development in *Gnetum* as well as in other gymnosperm species, to better hypothesize on the functional evolution of these genes.

Furthermore, the unique morphology of *Gnetum* ovules allows us to address hypotheses on the possible origin of the ovule. The three envelopes initiate in acropetal order: the outer

develops first from the lateral sides of the ovule primordia, then the middle, and then the integument shortly after, as a smooth outgrowth encircling the nucellus (Takaso and Bouman, 1986; Herr, 1995). The integument grows rapidly, surpassing the two envelopes forming the micropyle, and the exposed part forms multiple apical lobes (Fig. 1; Takaso and Bouman, 1986). These lobes seem to be similar to the integumentary lobes of Palaeozoic ovules (Herr, 1995). Interestingly, none of the genes studied here is expressed in the middle or outer envelopes of *Gnetum* ovules; most of them are restricted or strongly expressed in the apical region of the integument (i.e. *GneANT*, *GnmoKAN1/ 2*, *GnmoUCN* and *GnmoUCN2*; Figs 2–4).

Concerning the mechanisms that led to the evolution of ovules as a major synapomorphy of seed plants, three hypotheses are still debated and remain valid. (1) The integuments covering the megasporangia appeared as a new structure; this is known as the '*de novo* hypothesis' (Meeuse, 1966). (2) The integuments are the result of the fusion of vegetative structures, telomes, around the sporangium – the 'telome hypothesis' (De Haan, 1920; Walton, 1953); this hypothesis is supported by the fusion of integumentary lobes in the Palaeozoic ovules. (3) The integuments are the result of sterilization of sporangia around the only sporangium that remains functional – the 'synangial hypothesis' (Benson, 1904). The synangial hypothesis was accepted (Boesewinkel and Bouman, 1967; Takhtajan, 1981) and later modified (Kenrick and Crane, 1997) by following the vascular traces of the Palaeozoic ovules, thus also providing evidence for the 'neo-synangial hypothesis'. Palaeontological and morphological evidence so far seems equivocal for these three hypotheses. However, our studies in the integument genes in *G. gnemon* synthesized with previous studies in seedless plants (bryophytes, lycophytes and ferns) suggest two possible scenarios for ovule evolution. The first is that these genes were co-opted from the sporangium development network and became specific for integument development in angiosperms, which, is likely to have occurred, since plants are modular organisms (Fig. 5). In the second scenario the integuments are the result of fusion of integumentary lobes that once were fertile. The neo-synangial hypothesis is supported by fossil evidence and by the expression patterns of these genes in the micro-/ megasporangium and in the apical region of the integument (Yamada *et al.*, 2019). Moreover, anatomical development of ovules in Cycadales and the fossil record of *Genomosperma kidstonii* seem to support the synangial hypothesis (Rothwell and Scheckler, 1988; Sánchez-Tinoco and Engelman, 2004, 2005). The possibility that both scenarios have occurred is therefore plausible.

The envelopes of *Gnetum* do not seem to have genetic similarities with the integument of angiosperms, supporting the hypothesis that there is one integument in *Gnetum* and that the nature of the two additional envelops remains enigmatic. Differential expression analyses in dissected tissues from ovules are still required, which could reveal new candidate genes involved in the development of the different structures of seeds in *Gnetum*. Crucially, given the unique morphology of *Gnetum* ovules and the complex evolutionary history of these gene lineages, added to the uncertainty over monophyletic or polyphyletic origin of the ovule, it is difficult to extrapolate the results obtained here to other gymnosperms.

Finally, among seed plants the phylogenetic position of Gnetales has always been ambiguous. According to morphological data, Gnetales appears to be sister to angiosperms (Crane, 1985; Doyle and Donoghue 1986, 1992; Loconte and Stevenson, 1990), but the molecular data do not support this hypothesis. Gnetales, instead, seems to be sister to different groups of conifers, Pinaceae and non-Pinaceae (Ruhfel *et al.*, 2014; Wickett *et al.*, 2014; Forest *et al.*, 2018). Our data highlight the genetic differences among ovules of Gnetales and angiosperms, but to gain a better understanding of the evolution of this group of plants, revealing the similarities through developmental genetic studies in conifers is of great importance.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Figure S1: maximum likelihood analyses using selected sequences across land plants showing the phylogenetic position of *G. gnemon* homologues for *BELL1*, *KANADI* and *UNICORN*. Figure S2: maximum likelihood analyses using selected sequences of *AINTEGUMENTA* across seed plants. Figure S3: primer sequence and location for each sequence of interest used for the expression studies. Table S1: list of primer sequences used to make the probes.

FUNDING

This research was funded by the Eppley Foundation for Research to B.A.A.

ACKNOWLEDGEMENTS

The authors thank Dr Dennis Stevenson for helpful discussions and Marc Hachadourian, Director of the NYBG's Nolen Greenhouses, for access to the *Gnetum gnemon* collections and plant care. Special thanks to the three anonymous reviewers whose comments helped improve this manuscript. C.Z.-C. designed and performed the experiments. C.Z.-C. and B.A.A. wrote the manuscript. B.A.A obtained the funding. All authors read and approved the final manuscript. The authors declare that there is no conflict of interest.

LITERATURE CITED

Ambrose BA, Lerner DR, Ciceri P, Padilla CM, Yanofsky MF, Schmidt RJ. 2000. Molecular and genetic analyses of the silky1 gene reveal conservation in floral organ specification between eudicots and monocots. *Molecular Cell* **5**: 569–579.

Arnault G, Vialette ACM, Andres-Robin A, Fogliani B, Gâteblé G, Scutt CP. 2018. Evidence for the extensive conservation of mechanisms of ovule integument development since the most recent common ancestor of living angiosperms. *Frontiers in Plant Science* **9**: 1352.

Baker SC, Robinson-Beers K, Villanueva JM, Gaiser JC, Gasser CS. 1997. Interactions among genes regulating ovule development in *Arabidopsis thaliana*. *Genetics* **145**: 1109–1124.

Beccari O. 1877. Della organogenia dei flori feminei del *Gnetum gnemon* L. *Un Nouvo Giorno in Italia* **9**: 91–100.

Becker A, Bey M, Bürglin TR, Saedler H, Theissen G. 2002. Ancestry and diversity of BEL1-like homeobox genes revealed by gymnosperm

(*Gnetum gnemon*) homologs. *Development Genes and Evolution* **212**: 452–457.

Becker A, Saedler H, Theissen G. 2003. Distinct MADS-box gene expression patterns in the reproductive cones of the gymnosperm Gnetum gnemon. *Development Genes and Evolution* **213**: 567–572.

Bellaoui M, Pidkowich MS, Samach A, *et al.* 2001. The *Arabidopsis* BELL1 and KNOX TALE homeodomain proteins interact through a domain conserved between plants and animals. *Plant Cell* **13**: 2455–2470.

Bencivenga S, Simonini S, Benková E, Colombo L. 2012. The transcription factors BEL1 and SPL are required for cytokinin and auxin signaling during ovule development in *Arabidopsis*. *Plant Cell* **24**: 2886–2897.

Benson M. 1904. The origin of flowering plants. *New Phytologist* **3**: 49–51.

Boesewinkel FD, Bouman F. 1967. Integument initiation in *Juglans* and *Pterocarya*. *Acta Botanica Neerlandica* **16**: 86–101.

Bowman JL, Eshed Y, Baum SF. 2002. Establishment of polarity in angiosperm lateral organs. *Trends in Genetics* **18**: 134–141.

Brambilla V, Battaglia R, Colombo M, *et al.* 2007. Genetic and molecular interactions between BELL1 and MADS box factors support ovule development in *Arabidopsis*. *Plant Cell* **19**: 2544–2556.

Brenner ED, Stevenson D. 2006. Using genomics to study evolutionary origins of seeds. In: Williams C, ed. *Landscapes, genomics and transgenic conifers*. Dordrecht: Springer, 85–106.

Brown RH, Nickrent DL, Gasser CS. 2010. Expression of ovule and integument-associated genes in reduced ovules of Santalales. *Evolution & Development* **12**: 231–240.

Bui LT, Pandzic D, Youngstrom CE, *et al.* 2017. A fern AINTEGUMENTA gene mirrors BABY BOOM in promoting apogamy in *Ceratopteris richardii*. *Plant Journal* **90**: 122–132.

Bürglin TR. 1997. Analysis of TALE superclass homeobox genes (MEIS, PBC, KNOX, Iroquois, TGIF) reveals a novel domain conserved between plants and animals. *Nucleic Acids Research* **25**: 4173–4180.

Candela H, Johnston R, Gerhold A, Foster T, Hake S. 2008. The milkweed pod1 gene encodes a KANADI protein that is required for abaxial/adaxial patterning in maize leaves. *Plant Cell* **20**: 2073–2087.

Chamberlain CJ. 1935. *Gymnosperms. Structure and evolution*. Chicago: University of Chicago Press.

Colombo L, Battaglia R, Kater MM. 2008. *Arabidopsis* ovule development and its evolutionary conservation. *Trends in Plant Science* **13**: 444–450.

Crane PR. 1985. Phylogenetic analysis of seed plants and the origin of angiosperms. *Annals of the Missouri Botanical Garden* **72**: 716–793.

Crane PR, Friis EM, Pedersen KR. 1995. The origin and early diversification of angiosperms. *Nature* **374**: 27–33.

Crepet WL, Friis EM, Nixon KC. 1991. Fossil evidence for the evolution of biotic pollination. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* **333**: 187–95.

Dash M, Malladi A. 2012. The AINTEGUMENTA genes, MdANT1 and MdANT2, are associated with the regulation of cell production during fruit growth in apple (*Malus* × *domestica* Borkh.). *BMC Plant Biology* **12**: 98.

De Haan HRM. 1920. Contribution to the knowledge of the morphological value and the phylogeny of the ovule and its integuments. *Recueil des Travaux des botaniques Neerlandais* **17**: 219–324.

Dong YH, Yao JL, Atkinson RG, Putterill JJ, Morris BA, Gardner RC. 2000. MDH1: an apple homeobox gene belonging to the BEL1 family. *Plant Molecular Biology* **42**: 623–633.

Doyle JA, Donoghue MJ. 1986. Seed plant phylogeny and the origin of angiosperms: an experimental cladistic approach. *Botanical Review* **52**: 321–431.

Doyle JA, Donoghue MJ. 1992. Fossils and seed plant phylogeny reanalyzed. *Brittonia* **44**: 89–106.

Elliott RC, Betzner AS, Huttner E, *et al.* 1996. AINTEGUMENTA, an APETALA2-like gene of *Arabidopsis* with pleiotropic roles in ovule development and floral organ growth. *Plant Cell* **8**: 155–168.

Endress PK. 1996. Structure and function of female and bisexual organ complexes in Gnetales. *International Journal of Plant Sciences* **157**: S113–S125.

Endress PK. 2010. Flower structure and trends of evolution in eudicots and their major subclades. *Annals of the Missouri Botanical Garden* **97**: 541–583.

Endress PK. 2011. Angiosperm ovules: diversity, development, evolution. *Annals of Botany* **107**: 1465–1489.

Enugutti B, Kirchhelle C, Oelschner M, *et al.* 2012. Regulation of planar growth by the *Arabidopsis* AGC protein kinase UNICORN. *Proceedings of the National Academy of Sciences of the USA* **109**: 15060–15065.

Enugutti B, Kirchhelle C, Schneitz K. 2013. On the genetic control of planar growth during tissue morphogenesis in plants. *Protoplasma* **250**: 651–661.

Eshed Y, Baum SF, Perea JV, Bowman JL. 2001. Establishment of polarity in lateral organs of plants. *Current Biology* **21**: 1251–1260.

Eshed Y, Izhaki A, Baum SF, Floyd SK, Bowman JL. 2004. Asymmetric leaf development and blade expansion in *Arabidopsis* are mediated by KANADI and YABBY activities. *Development* **131**: 2997–3006.

Favaro R, Pinyopich A, Battaglia R, *et al.* 2003. MADS-box protein complexes control carpel and ovule development in *Arabidopsis*. *Plant Cell* **15**: 2603–2611.

Ferrandiz C, Pelaz S, Yanofsky MF. 1999. Control of carpel and fruit development in *Arabidopsis*. *Annual Review of Biochemistry* **99**: 321–354.

Figueiredo DD, Köhler C. 2014. Signalling events regulating seed coat development. *Biochemical Society Transactions* **42**: 358–363.

Figueiredo DD, Köhler C. 2016. Bridging the generation gap: communication between maternal sporophyte, female gametophyte and fertilization products. *Current Opinion in Plant Biology* **29**: 16–20.

Flynn P, Wongdagger M, Zavar M, Dean NM, Stokoe D. 2000. Inhibition of PDK-1 activity causes a reduction in cell proliferation and survival. *Current Biology* **10**: 1439–1442.

Forest F, Moat J, Baloch E, *et al.* 2018. Gymnosperms on the EDGE. *Scientific Reports* **8**: 6053.

Gasser CS, Skinner DJ. 2019. Development and evolution of the unique ovules of flowering plants. *Current Topics in Developmental Biology* **131**: 373–399.

Gasser CS, Broadhvest J, Hauser BA. 1998. Genetic analysis of ovule development. *Annual Review of Plant Physiology and Plant Molecular Biology* **49**: 1–24.

Gehring WJ, Müller M, Affolter M, *et al.* 1990. The structure of the homeodomain and its functional implications. *Trends in Genetics* **1**: 323–329.

Gross-Hardt R, Lenhard M, Laux T. 2002. WUSCHEL signaling functions in interregional communication during *Arabidopsis* ovule development. *Genes & Development* **16**: 1129–1138.

Herr JM Jr. 1995. The origin of the ovule. *American Journal of Botany* **82**: 547–564.

Hollander JL, Vander Wall SB. 2009. Dispersal syndromes in North American *Ephedra*. *International Journal of Plant Sciences* **170**: 323–330.

Hollander JL, Vander Wall SB, Baguley JG. 2010. Evolution of seed dispersal in North American *Ephedra*. *Evolutionary Ecology* **24**: 333–345.

Horst NA, Katz A, Pereman I, Decker EL, Ohad N, Reski R. 2016. A single homeobox gene triggers phase transition, embryogenesis and asexual reproduction. *Nature Plants* **2**: 15209.

Hosoda K, Imamura A, Katoh E, *et al.* 2002. Molecular structure of the GARP family of plant Myb-related DNA binding motifs of the *Arabidopsis* response regulators. *Plant Cell* **14**: 2015–2029.

Huang T, Harrar Y, Lin C, *et al.* 2014. *Arabidopsis* KANADI1 acts as a transcriptional repressor by interacting with a specific cis-element and regulates auxin biosynthesis, transport, and signaling in opposition to HD-ZIPIII factors. *Plant Cell* **26**: 246–262.

Ickert-Bond SM. 2003. *Systematics of New World* Ephedra *L.(Ephedraceae): integrating morphological and molecular data.* PhD Thesis, Arizona State University, USA.

Ickert-Bond SM, Rydin C. 2011. Micromorphology of the seed envelope of *Ephedra* L.(Gnetales) and its relevance for the timing of evolutionary events. *International Journal of Plant Sciences* **172**: 36–48.

Izhaki A, Bowman JL. 2007. KANADI and class III HD-Zip gene families regulate embryo patterning and modulate auxin flow during embryogenesis in *Arabidopsis*. *Plant Cell* **19**: 495–508.

Kelley DR, Gasser CS. 2009. Ovule development: genetic trends and evolutionary considerations. *Sexual Plant Reproduction* **22**: 229–234.

Kelley DR, Skinner DJ, Gasser CS. 2009. Roles of polarity determinants in ovule development. *Plant Journal* **57**: 1054–1064.

Kelley DR, Arreola A, Gallagher TL, Gasser CS. 2012. ETTIN (ARF3) physically interacts with KANADI proteins to form a functional complex essential for integument development and polarity determination in *Arabidopsis*. *Development* **139**: 1105–1109.

Kenrick P, Crane PR. 1997. The origin and early evolution of plants on land. *Nature* **389**: 33–39.

Kerstetter RA, Bollman K, Taylor RA, Bomblies K, Poethig RS. 2001. KANADI regulates organ polarity in *Arabidopsis*. *Nature* **411**: 706–709.

Kim S, Soltis PS, Wall K, Soltis DE. 2006. Phylogeny and domain evolution in the APETALA2-like gene family. *Molecular Biology and Evolution* **23**: 107–120.

Klucher KM, Chow H, Reiser L, Fischer RL. 1996. The AINTEGUMENTA gene of *Arabidopsis* required for ovule and female gametophyte development is related to the floral homeotic gene APETALA2. *Plant Cell* **8**: 137–153.

Kramer EM, Jaramillo MA, Di Stilio VS. 2004. Patterns of gene duplication and functional evolution during the diversification of the AGAMOUS subfamily of MADS box genes in angiosperms. *Genetics* **166**: 1011–1023.

Laux T, Mayer KF, Berger J, Jürgens G. 1996. The WUSCHEL gene is required for shoot and floral meristem integrity in *Arabidopsis*. *Development* **122**: 87–96.

Leon-Kloosterziel KM, Keijzer CJ, Koornneef M. 1994. A seed shape mutant of *Arabidopsis* that is affected in integument development. *Plant Cell* **6**: 385–392.

Loconte H, Stevenson DW. 1990. Cladistics of the Spermatophyta. *Brittonia* **42**: 197–211.

Lora J, Hormaza JI, Herrero M. 2015. Transition from two to one integument in *Prunus* species: expression pattern of INNER NO OUTER (INO), ABERRANT TESTA SHAPE (ATS) and ETTIN (ETT). *New Phytologist* **208**: 584–595.

Losa A, Colombo M, Brambilla V, Colombo L. 2010. Genetic interaction between AINTEGUMENTA (ANT) and the ovule identity genes SEEDSTICK (STK), SHATTERPROOF1 (SHP1) and SHATTERPROOF2 (SHP2). *Sexual Plant Reproduction* **23**: 115–121.

Manchado-Rojo M, Weiss J, Egea-Cortines M. 2014. Validation of Aintegumenta as a gene to modify floral size in ornamental plants. *Plant Biotechnology Journal* **12**: 1053–1065.

Martens P. 1971. *Les Gnétophytes. Handbuch der Pflanzenanatomie, XII (2). 2nd edn*. Berlin: Borntraeger.

Mayer KF, Schoof H, Haecker A, Lenhard M, Jürgens G, Laux T. 1998. Role of WUSCHEL in regulating stem cell fate in the *Arabidopsis* shoot meristem. *Cell* **95**: 805–815.

McAbee JM, Hill TA, Skinner DJ, *et al.* 2006. ABERRANT TESTA SHAPE encodes a KANADI family member, linking polarity determination to separation and growth of *Arabidopsis* ovule integuments. *Plant Journal* **46**: 522–531.

Meeuse ADJ. 1966. *Fundamentals of phytomorphology.* New York: The Ronald Press Company.

Miller MA. Pfeiffer W, Schwartz T. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: *Proceedings of the Gateway Computing Environments Workshop (GCE), 14 November 2010*, New Orleans, LA, 1–8.

Miller MA, Pfeiffer W, Schwartz T. 2012. The CIPRES Science Gateway: enabling high-impact science for phylogenetics researchers with limited resources. In: Proceedings of the 1st Conference of the Extreme Science and Engineering Discovery Environment: Bridging from the Extreme to the Campus and Beyond, 1–8.

Mizukami Y, Fischer RL. 2000. Plant organ size control: AINTEGUMENTA regulates growth and cell numbers during organogenesis. *Proceedings of the National Academy of Sciences of the USA* **97**: 942–947.

Modrusan Z, Reiser L, Feldmann KA, Fischer RL, Haughn GW. 1994. Homeotic transformation of ovules into carpel-like structures in *Arabidopsis*. *Plant Cell* **6**: 333–349.

Müller J, Wang Y, Franzen R, Santi L, Salamini F, Rohde W. 2001. In vitro interactions between barley TALE homeodomain proteins suggest a role for protein-protein associations in the regulation of Knox gene function. *Plant Journal* **27**: 13–23.

Nardmann J, Werr W. 2012. The invention of WUS-like stem cell-promoting functions in plants predates leptosporangiate ferns. *Plant Molecular Biology* **78**: 123–134.

Nardmann J, Reisewitz P, Werr W. 2009. Discrete shoot and root stem cell-promoting WUS/WOX5 functions are an evolutionary innovation of angiosperms. *Molecular Biology and Evolution* **26**: 1745–1755.

Neumann U, Hay A. 2020. Seed coat development in explosively dispersed seeds of *Cardamine hirsuta*. *Annals of Botany* **126**: 39–59.

Nole-Wilson S, Krizek BA. 2006. AINTEGUMENTA contributes to organ polarity and regulates growth of lateral organs in combination with YABBY genes. *Plant Physiology* **141**: 977–987.

Pabón-Mora N, Wong GK, Ambrose BA. 2014. Evolution of fruit development genes in flowering plants. *Frontiers in Plant Science* **5**: 300.

Pinyopich A, Ditta GS, Savidge B, *et al.* 2003. Assessing the redundancy of MADS-box genes during carpel and ovule development. *Nature* **424**: 85–88.

Ray A, Robinson-Beers K, Ray S, *et al.* 1994. *Arabidopsis* floral homeotic gene BELL (BEL1) controls ovule development through negative regulation of AGAMOUS gene (AG). *Proceedings of the National Academy of Sciences of the USA* **91**: 5761–5765.

Reinhart BJ, Liu T, Newell NR, *et al.* 2013. Establishing a framework for the Ad/abaxial regulatory network of *Arabidopsis*: ascertaining targets of class III homeodomain leucine zipper and KANADI regulation. *Plant Cell* **25**: 3228–3249.

Reiser L, Modrusan Z, Margossian L, *et al.* 1995. The BELL1 gene encodes a homeodomain protein involved in pattern formation in the *Arabidopsis* ovule primordium. *Cell* **83**: 735–742.

Riechmann JL, Heard J, Martin G, *et al.* 2000. *Arabidopsis* transcription factors: genome-wide comparative analysis among eukaryotes. *Science* **290**: 2105–2110.

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

Rothwell, GW, Scheckler SE. 1988. Biology of ancestral gymnosperms. In: Beck CD, ed. *Origin and evolution of gymnosperms*. New York: Columbia University Press.

Rudall PJ. 2021. Evolution and patterning of the ovule in seed plants. *Biological Reviews* **96**: 943–960.

Ruhfel BR, Gitzendanner MA, Soltis PS, Soltis DE, Burleigh JG. 2014. From algae to angiosperms-inferring the phylogeny of green plants (Viridiplantae) from 360 plastid genomes. *BMC Evolutionary Biology* **14**: 23.

Rydin C, Pedersen KR, Crane PR, Friis EM. 2006. Former diversity of *Ephedra* (Gnetales): evidence from Early Cretaceous seeds from Portugal and North America. *Annals of Botany* **98**: 123–140.

Rydin C, Khodabandeh A, Endress PK. 2010. The female reproductive unit of *Ephedra* (Gnetales): comparative morphology and evolutionary perspectives. *Botanical Journal of the Linnean Society* **163**: 387–430.

Sánchez-Tinoco MY, Engleman EM. 2004. Seed coat anatomy of Ceratozamia mexicana (Cycadales). *Botanical Review* **70**: 24–38.

Sánchez-Tinoco MY, Engleman EM. 2005. The vascularization of the seed of Ceratozamia mexicana (Zamiaceae). In: *Proceedings of Cycad 2005*. New York: New York Botanical Garden Press, 223–235.

Schneitz K, Hülskamp M, Pruitt RE. 1995. Wild-type ovule development in *Arabidopsis thaliana*: a light microscope study of cleared whole-mount tissue. *The Plant Journal* **7**: 731–749.

Schneitz K, Hülskamp M, Kopczak SD, Pruitt RE. 1997. Dissection of sexual organ ontogenesis: a genetic analysis of ovule development in *Arabidopsis thaliana*. *Development* **124**: 1367–1376.

Scholz S, Pleßmann J, Enugutti B, Hüttl R, Wassmer K, Schneitz K. 2019. The AGC protein kinase UNICORN controls planar growth by attenuating PDK1 in *Arabidopsis thaliana*. *PLoS Genetics* **15**: e1007927.

Schoof H, Lenhard M, Haecker A, Mayer KF, Jürgens G, Laux T. 2000. The stem cell population of *Arabidopsis* shoot meristems is maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. *Cell* **100**: 635–644.

Sieber P, Gheyselinck J, Gross-Hardt R, Laux T, Grossniklaus U, Schneitz K. 2004. Pattern formation during early ovule development in *Arabidopsis thaliana*. *Developmental Biology* **273**: 321–334.

Skinner DJ, Hill TA, Gasser CS. 2004. Regulation of ovule development. *Plant Cell* **16** (Suppl): S32–S45.

Skinner DJ, Brown RH, Kuzoff RK, Gasser CS. 2016. Conservation of the role of INNER NO OUTER in development of unitegmic ovules of the Solanaceae despite a divergence in protein function. *BMC Plant Biology* **16**: 143.

Stamatakis A, Hoover P, Rougemont J. 2008. A rapid bootstrap algorithm for the RAxML web servers. *Systematic Biology* **57**: 758–771.

Strasburger E. 1879. *Die Angiospermen und die Gymnospermen.* Jena: Fisher.

Takaso T. 1984. Structural changes in the apex of the female strobilus and the initiation of the female reproductive organ (ovule) in Ephedra distachya L. and E. equisetina Bge. *Acta Botanica Neerlandica* **33**: 257–266.

Takaso T. 1985. A development study of the integument in gymnosperms. 3. *Ephedra distachya* L. and *E. equisetina* Bge. *Acta Botanica Neerlandica* **34**: 33–48.

Takaso T, Bouman F. 1986. Ovule and seed ontogeny in Gnetum gnemon L. *Botanical Magazine. Tokyo* **99**: 241–266.

Takhtajan AL. 1981. Flowering plants: origin and dispersal. Edinburgh, Scotland: Oliver and Boyd, 310–313.

Tomlinson PB, Braggins JE, Rattenbury JA. 1991. Pollination drop in relation to cone morphology in Podocarpaceae: a novel reproductive mechanism. *American Journal of Botany* **78**: 1289–1303.

Vasco A, Smalls TL, Graham SW, *et al.* 2016. Challenging the paradigms of leaf evolution: Class III HD-Zips in ferns and lycophytes. *New Phytologist* **212**: 745–758.

Walton J. 1953. Evolution of the ovule in pteridosperms. *Nature* **172**: 435–436.

Wickett NJ, Mirarab S, Nguyen N, *et al.* 2014. Phylotranscriptomic analysis of the origin and early diversification of land plants. *Proceedings of the National Academy of Sciences of the USA* **111**: E4859–E4868.

Wu CC, Li FW, Kramer EM. 2019. Large-scale phylogenomic analysis suggests three ancient superclades of the WUSCHEL-RELATED HOMEOBOX transcription factor family in plants. *PloS one* **14**: e0223521.

Yamada T, Ito M, Kato M. 2003. Expression pattern of INNER NO OUTER homologue in *Nymphaea* (water lily family, Nymphaeaceae). *Development Genes and Evolution* **213**: 510–513.

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

Yamada T, Sasaki Y, Hashimoto K, Nakajima K, Gasser CS. 2016. CORONA, PHABULOSA and PHAVOLUTA collaborate with BELL1 to confine WUSCHEL expression to the nucellus in *Arabidopsis* ovules. *Development* **143**: 422–426.

Yamada T, Sasaki Y, Sakata K, Gasser CS. 2019. Possible roles of BELL 1 and class III homeodomain-leucine zipper genes during integument evolution. *International Journal of Plant Sciences* **180**: 623–631.

Youngstrom CE, Geadelmann LF, Irish EE, Cheng CL. 2019. A fern WUSCHEL-RELATED HOMEOBOX gene functions in both gametophyte and sporophyte generations. *BMC Plant Biology* **19**: 416.

Zhang GH, Xu Q, Zhu XD, Qian Q, Xue HW. 2009. SHALLOT-LIKE1 is a KANADI transcription factor that modulates rice leaf rolling by regulating leaf abaxial cell development. *Plant Cell* **21**: 719–735.

Zumajo-Cardona C, Ambrose BA. 2020. Phylogenetic analyses of key developmental genes provide insight into the complex evolution of seeds. *Molecular Phylogenetics and Evolution* **147**: 106778.

Zumajo-Cardona C, Vasco A, Ambrose BA. 2019. The evolution of the KANADI gene family and leaf development in lycophytes and ferns. *Plants* **8**: 313.