Organogenesis and Vasculature of *Anaxagorea* and its Implications in the Integrated Axial-Foliar Origin of the Angiosperm Carpel

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Running Title: The Axial-Foliar Origin of Carpel

Abstract

The carpel is the definitive structure of angiosperms and the origin of the carpel is of great significance to the phylogenetic origin of angiosperms. The traditional view has been that angiosperm carpels emerged from structures similar to macrosporophylls of pteridosperms or Bennettitales, which bear ovules on the surface of foliar organs. Conversely, according to other perspectives, carpels originated from foliar appendages enclosing the ovule-bearing axis. One of the key distinctions between the two conflicting views lies in whether the ovular axis is involved in the evolution of the carpel. The aim of the present study was to investigate whether the axial homologs remain in the carpel of extant angiosperms, and, in turn, reveal the integrated axial-foliar nature of carpel. Two *Anaxagorea* species were used for organogenesis and comparative anatomical studies owing to their outstanding and unusually long carpel stipes. The continuous change of vascular bundles at the carpel stipe at different developmental stages is described here in detail. Organogenesis revealed that the carpel stipe of *Anaxagorea* emerges at the early stage of carpel development. Vascular bundles at the base of *Anaxagorea* carpel are a set of discrete ring-arranged collateral bundles that branch out at the upper portion into two sets of ring-arranged collateral bundles below each ovule. The ring-arranged collateral bundles are clear morphological evidence of the existence of axial homologs in the carpel, and, therefore, support the perspective that carpels originated from the integration of the ovular axis and foliar parts. This finding could prompt the reconsideration of how the bract-bracteole-terminal ovule system in gymnosperms evolved into an angiosperm carpel.

Key words: *Anaxagorea*; axial homologs; carpel; organogenesis; origin of angiosperms; vascular anatomy.

INTRODUCTION

Angiosperms, the flowering plants, account for a considerable proportion of the plants visible to modern human society. Both the beautiful flowers and the delicious fruits are intertwined with human life. The term "angiosperm" is derived from the Greek words angeion, meaning "container," and sperma, meaning "seed." Therefore, the carpel, a "seeds container," is the definitive characteristic in angiosperms. The carpel, which protectively surrounds the ovules by closure and sealing along their rims or flanks, is an angiosperm-specific female reproductive feature in flowers and is the basic unit of the gynoecium (Dunal, 1817; Robinson-Beers, 1992; Endress, 2015). The evolution of the carpel set angiosperms apart from other seed plants, which developed ovules that were exposed to the air. Since the time of Darwin, elucidating the origin of angiosperms and their evolutionary success has been a primary goal in plant science. Scientists have attempted to identify the potential ancestors of angiosperms through phylogenetic analyses based on fossil, morphological, and molecular data. In such efforts, particular emphasis has been placed on assessing which ovule-bearing structures of various seed plants could be transformed into carpels.

The history of exploring the origin of carpel has been tortuous. Conflicting theories with regard to flower origins have provided varied explanations and influenced hypotheses on the phylogenetic relationships among angiosperms and other seed plant lineages. The traditional phyllosporous origin hypothesis suggests that angiosperm carpels were derived from structures similar to macrosporophylls of Bennettitales, which bear ovules on the margins of foliar organs (Arber and Parkin, 1907; Eyde, 1975; Cronquist, 1988; Takhtajan, 1991). Conversely, according to the stachyosporous origin theory, the carpel originated from a compound shoot with the integration of the ovular axis and the foliar appendage. The placenta with ovules is homologous with a female short-shoot in gymnosperms with distally placed ovules, each with scales forming the outer integument (Stebbins, 1974; Retallack and Dilcher, 1981a; Crane, 1985; Doyle and Donoghue, 1986; Nixon *et al.*, 1994; Hickey and Taylor, 1996; Wang, 2010, 2018). In addition, according to the Caytonialean hypothesis, the cupule wall of seed ferns is homologous to the outer integument of angiosperm ovules and that expansion and folding of the cupule-bearing axis are precursors to the carpel (Doyle, 1978, 2006, 2008). Another theory posits that the carpel evolved via the ectopic expression of ovules on a phyllome (i.e., the Mostly Male theory [Frohlich and Parker, 2000]).

However, since no sister group of the angiosperms has been identified, the origin of the carpel remains a mystery. Progress has been made in morphological and functional genetics studies among angiosperms. In angiosperms, the placenta and ovary wall are served by distinct vascular bundles (Guo et al., 2013; Liu et al., 2014; Guo et al., 2017; Zhang et al., 2017) and are regulated by different genes associated with branch and leaf organs, respectively (Roe et al., 1997; Skinner et al., 2004; Mathews and Kramer, 2012). In distributed lines of angiosperms, the ovule primordium originates in the axillary position between the flower apex and carpel

wall (e.g., *Gyrostemon* [Hufford, 1996]; *Illicium* [Zhang et al., 2019]; *Phytolacca* [Zhang et al., 2018]). These are key clues with regard to whether the placenta and the carpel wall have different evolutionary origins; however, more solid evidence is required. According to the above-mentioned hypotheses, it is critical to find clear evidence for the existence of axial homologs in the carpel. Based on the premise that the carpel wall is certainly derived from foliar homologs, on condition that the axial homologs in the carpel are determined, the carpel would more likely be derived from an ovule-bearing axis fused with the leaf-like structure than from the megasporophyll.

To present more reliable evidence for the existence of axial homologs, the carpel of *Anaxagorea* (Annonaceae) was selected for organogenesis and vascular anatomic examination. Annonaceae represents one of the largest families in the Magnoliales, which is one of the most important lineages in the early radiation of angiosperms (Sauquet et al., 2003), while *Anaxagorea* is the most basal genus in Annonaceae (Doyle and le Thomas, 1996; Doyle et al., 2004; Chatrou et al., 2012; Chatrou et al., 2018), which are found in dimly-lit riparian zones in forest slope and understory habitats. *Anaxagorea* have simple leaves that are arranged alternately in two rows along the stems and flowers often have whorled phyllotaxis. Gynoecia are apocarpous (free carpels) throughout a life history (Deroin, 1988). Each carpel in *Anaxagorea* has a notable long stipe (Endress and Armstrong, 2011), which is one of the key characteristics of the genus. Morphological features in *Anaxagorea* are relatively primitive in the angiosperms and the presence of the notable carpel stipe makes it possible to determine whether there are “axial homologs” in the carpel through tissue sectioning.

MATERIALS AND METHODS

Study Species and Sites

Flower samples of *Anaxagorea luzonensis* and *Anaxagorea javanica* were collected in July 2017 from Diaoluo Mountain (Hainan, China) and the Xishuangbanna Tropical Botanical Garden (Yunnan, China), respectively. The former usually has two to four carpels, whereas the latter has a multicarpellate gynoecium.

Scanning Electron Microscopy and Paraffin Sectioning

Flower samples were collected at different floral stages (from early bud to young fruit). Gynoecia were isolated from the other parts of the flower and preserved in formalin–acetic acid–70% alcohol (5:5:90, v/v). The fixed material were dehydrated through an alcohol series (50% to 100%). To delineate the structure and development of the carpel, some carpels were removed from gynoecia and passed through an iso-pentanol acetate series (SCR, Shanghai, China), critically point-dried, sputter-coated with gold, and observed and photographed under a scanning electron microscope (Tescan VEGA-3-LMU, Brno, Czech Republic). To illustrate the vasculature of the carpel, some carpels were embedded in paraffin, sectioned serially into 10–12-μm thick sections, and then stained with Safranin O and Fast Green. Complete transverse and longitudinal sections were examined and photographed

under a light microscope (Olympus BX-43-U, Tokyo, Japan) and bright-field microscopy.

RESULTS

Carpel Organogenesis

In *A. luzonensis* and *A. javanica*, carpels are completely free from each other (**Figures 1G, 2A**). Each carpel consists of a plicate zone (**Figures 1G, 3B–H**), a very short ascidiate zone (**Figure 2C**), and a conspicuous long stipe (**Figures 1G, 3I–M**). Carpel primordia are almost initiated hemispherically (**Figure 1A**). Carpel stipe originates at the basal part in the early stages of carpel development (**Figures 1C, J**), remains elongate, accounts for roughly a quarter of the length of the carpel at anthesis (**Figure 1G**), and continues to elongate during the fruit-stage. Continuous growth of the flanks on the ventral side of the young carpel triggers the early closure. The closure does not extend to the bottom of the carpel (**Figure 1D**). Subsequently, the dorsal part of each carpel thickens markedly and the stigma forms (**Figures 1E, F**). At anthesis, carpels are widest at the basal part with an arch on the abaxial side (**Figure 1G**). Each carpel has two lateral ovules with the placentae at the base of the ovary (**Figures 2F, M, 3A**).

Carpel Vasculature

The main vascular bundles in the *Anaxagorea* carpel are distinct as a dorsal bundle, a pair of ventral bundles, a pair of ovule bundles, and several lateral bundles (**Figures 2F, M, 3A, F**). In the present study, vascular systems are described from the base to the carpel for easier comprehension of vascular transformation (**Figure 3**). At the base of each carpel stipe, discrete collateral (a collateral bundle has an adaxial xylem and an abaxial phloem) bundles form a ring (hereafter abbreviated as ring-arranged collateral bundles, RACBs) around the pith-like cell groups (**Figures 2A, I, 3M**). Slightly on the upper part of the carpel stipe, the discrete bundles tend to join adjacent bundles in the phloem (**Figure 3L**), subsequently separate, and finally gather into three groups: a dorsal bundle and two sets of lateral bundle complexes (LBCs, **Figure 3K**). Each set of LBCs is assembled into an amphicribral (an amphicribral bundle has its xylem surrounded by the phloem) bundle in the young carpel (**Figure 2B**). In a mature carpel, each set of LBCs is assembled as a set of RACBs; therefore, there are two sets of RACBs in one carpel (**Figures 2E, K, 3J**). Below each placenta, each set of RACBs (or amphicribral bundle) is transformed into a set of “C”-shaped bundle complexes (CBCs), from which several collateral bundles separate from each set of CBCs, and merge to form an amphicribral bundle, which is maintained separate from other bundles (**Figures 2C, L, 3G–I**). Each set of CBCs allocates an ovule bundle into each ovule and other bundles into the carpel wall, where the ventral bundles are also separated (**Figures 2D, F, M, 3E–F**). In the cross-section of the ovary locule, both ventral bundles and ovule bundles are amphicribral (**Figures 2G, H**). In the carpel wall, lateral bundles are pinnately arranged collateral bundles, which are connected to form mesh-like complexes with poorly differentiated strands (**Figures 2M, 3D, E**). The dorsal bundle is also connected to lateral bundles by poorly

differentiated strands (**Figures 2D, 3F**). Upon the ovule locule, bundles in the carpel wall join each other with the xylem and phloem and finally diminish (**Figures 3B, C**).

DISCUSSION

The present study investigated the organogenesis and the changes in the vascular bundle at different development stages of the carpel stipe in *Anaxagorea*. The unique arrangement of vascular bundles in the carpel stipe of *Anaxagorea* suggests there are axial homologous in the carpels of modern angiosperms and represent a critical step toward the understanding of how carpels evolved from female reproductive secondary shoots and sterile bracts.

Peltate carpels have been suggested to be plesiomorphic in Annonaceae (Deroin, 1988; Igersheim and Endress, 1997; Surveswaran et al., 2010; Couvreur et al., 2011). In addition, in some studies, *Anaxagorea* carpels have been reported to have a short ascidiate zone (Deroin, 1988), while they have been described as completely plicate in other studies (Endress and Armstrong, 2011). In the present study, organogenesis reveals that the carpel stipe of *A. luzonensis* and *A. javanica* emerges at the early stages of carpel development and continues to elongate with the development of the carpel, which is different from the course of development of mericarp stipes of other Annonaceae plants, the latter of which has the ovuliferous upper ovary parts are develop first, and then sterile lower stipes emerge later (e.g., *Cananga*, *Uvari*, *Xylopia* [Deroin, 1999]). In the flower stage, the ventral slit may continue for a distance along the ovule locule and terminate very close to the base of the ovule locule. Such carpel variants of this genus maysuggest transformation from peltate carpels to plicate carpels. The carpel stipe in *Anaxagorea* provides a buffer space for the drastic changes in structure in the vascular bundle at the base of the carpel, whereas in most angiosperms with apocarpy, the base of the carpel is very close to the placenta and the vascular bundle in this part tends to be simplified (e.g., *Michelia* [Tucker, 1961]; *Sagittaria* [Kaul, 1967]; *Drimys* [Tucker, 1975]; *Illicium* [Robertson and Tucker, 1979]; *Brasenia* [Endress, 2005]).

In Annonaceae, carpels are usually supplied by three bundles from the cortical vascular system (Eames, 1961; Deroin and Norman, 2016; Deroin and Bidault, 2017). However, in *A. luzonensis* and *A. javanica*, the number of vascular bundles entering the carpel is far more than three and the vascular bundles are arranged in a radiosymmetric pattern. Radiosymmetric vasculature is a universal feature in the stems of vascular plants (Metcalfe and Chalk, 1979; Evert, 2006; Beck, 2010; McKown and Dengler, 2010; Evert and Eichhorn, 2011). In stem cross-sections in most angiosperms other than monocots, discrete collateral bundles form single rings or continuous hollow cylinders around the pith (e.g., Caprifoliaceae; Leguminosae; Tiliaceae; Ulmaceae). Conversely, in leaves, vascular bundles are laterally organized in most seed plants, and the largest collateral bundles often extend along the long axis of the leaf as a midvein from which smaller veins diverge laterally (e.g., Pinaceae; Gnetaceae; Magnoliaceae; Gramineae; Brassicaceae). This is observed even in branching veins in *Ginkgo*, where each of the veins is a single collateral bundle. In the

cross-sections of the *Anaxagorea* carpel, the RACBs at the basal part act in a manner consistent with the typical form of the stem vascular anatomy of most angiosperms. In the distal region, a dorsal bundle is separated from the basal RACBs and the remainder are gathered into two sets of amphicribral bundles below each placenta, which are developed into two sets of RACBs in the more mature carpel. The vasculature transition could be key indicators that (1) the basal part of the *Anaxagorea* carpel stipe is an axial homolog in nature, and (2) the carpel is an axial-foliar integration that originates from the ovular axis with its bract. The branching of RACBs in each carpel seems to be evidence of the existence of axial homologs in the carpel because the foliar homologs (lateral organs) cannot form such a topology by coiling transformation since the topological structure of the lateral organized bundles is equivalent to a curve, and the curved surface can only form a one-way tunnel (like macaroni pasta) by coiling. However, the branching RACBs (one set of RACBs branch into two sets of RACBs) in the carpel of *Anaxagorea* are analogous to three-way tunnels.

Development of amphicribral bundles into RACBs was observed in *A. luzonensis* carpels at different stages. The ovule bundles were also amphicribral, and were separated from the CBCs. Amphicribral bundles are frequently observed in small branches of early land plants, in monocots, or in young branches of dicots as simplification and combination of stem bundles (Fahn, 1990). In *Magnolia* (Liu et al., 2014) and *Michelia* carpels (Zhang et al., 2017), amphicribral bundles were observed supplying the ovules and emerged from cortical amphicribral bundles. In *Actinidia* (Guo et al., 2013) and *Dianthus* pistils (Guo et al., 2017), amphicribral bundles are reported in the placenta and are distinct from the collateral bundles in the carpel wall. Nevertheless, amphicribral bundles seem widespread in placenta and funiculus of angiosperms (e.g., *Papaver* [Kapoor, 1973], *Psoraleae* [Lersten and Don, 1966], *Drimys* [Tucker, 1975], *Nicotiana* [Dave et al., 1981], *Whytockia* [Wang and Pan, 1998], *Pachysandra* [Von Balthazar and Endress, 2002]). In addition to the above findings, the amphicribral bundles in the placenta could be considered RACBs simplification if the ovule and placenta in the carpel originate from the ovular axis.

The challenge of deciphering the relationship between angiosperms and other seed plant lineages has persisted for decades. Taylor (1991) once set a goal to research the origin of angiosperms, that is, the correlation between carpels in angiosperms and reproductive structures in other seed plants. Gnetophytes have been one of the candidate angiosperm ancestors based on the results of comparisons of the female reproductive shoot of *Ephedra* with angiosperm carpel (Hickey and Taylor, 1996); however, the potential relationship has since been refuted based on the results of molecular phylogenetic studies (Hajibabaei et al., 2006; Wickett et al., 2014; Wan et al., 2018). To date, the leaf origin theory remains the mainstream perspective with regard to the origin of carpels. However, as Endress (2019) interpreted, the ‘axis’ and ‘leaf’ are parts belonging to different levels of the structural hierarchy of a shoot (surface vs. internal region), they cannot logically be delimited from each other. Consequently, any leaf structure has to be attached to the axis, and the entire carpel

should be considered a compound shoot since the outer integument of the ovule is a foliar organ. The Unifying Theory (Wang, 2010, 2018) suggests that angiosperm carpels are derived from the sterile bracts (formed carpel wall) attached to the ovule-bearing secondary shoot (formed ovule/placenta) of cordaitaleans, a common ancestor of conifers and gnetophytes (Eames, 1952; Bold, 1973; Hickey and Taylor, 1996; Doyle, 2008). Considering cordaitaleans as the common ancestor of angiosperms seems to facilitate the resolving of the contradiction between the similar morphological characters and the phylogenetic distance between angiosperms and gnetophytes. Recently, by comparing the morphology of the many types of ovulate structures in glossopterids, McLoughlin and Prevec (2019) showed that the cupule of glossopterids, a group of extinct seed plants which are affiliated with angiosperms (Plumstead, 1956; Retallack and Dilcher, 1981b; Melville, 1983), is not a leaf-like structure but a modified branch with numerous uniovulate sporophylls. In addition, the fertilizers of glossopterids have been borne on loose strobili in which individual polysperms represent fertile cladodes of diverse morphologies subtended by a vegetative leaf or bract, which are similar to the condensed bract/ovuliferous scale complexes of conifers and cordaitaleans. Since such potential ancestors including cordaitaleans are fossils that still have numerous characters that are unclear or require further validation, more pieces of evidence are required before comprehensive phylogenetic analyses of seed plants can be undertaken. More clues with regard to the origin of the carpel in angiosperms could facilitate such studies. The branching RACBs of *Anaxagorea* carpels imply that the carpel is comparable to a compound shoot and prompt the reconsideration of the phylogenetic relationships among angiosperms, gnetophytes, and Cordaitales (**Figure 4**).

Overall, our results provide reliable evidence that the placenta with the ovule, and the carpel wall have distinct evolutionary origins, which supports the hypothesis that carpels have originated from sterile bracts attached to ovule-bearing shoots. However, the investigations in the present study were limited to a single genus. We hypothesize that the axial homologs are a regular feature in the angiosperm carpel. However, such homologs may have diverse characteristics such as the RACBs and the amphicribral bundles because the vascular bundle in the carpel is often simplified. Future studies should conduct more extensive studies, with a focus on early-diverging angiosperm groups with carpel stipe, such as Cabomboideae, Illicieae, and Schisandreae, to test the validity of the hypothesis. Nature selects simple mechanisms or modules as building blocks for complex systems, and then employing such modules repeatedly in other systems. The emergence of axial homologs in the carpel seem to embody this principle. Notably, the term “carpel” was first defined in the Annonaceae family by Dunal (1817), two hundred years ago. Today, new evidence has prompted to us to reexamine this large family anew.

Elucidating the origin of angiosperms requires the integration of morphological and molecular evidence from a wide range of fossil and extant seed plant data. For example, a comprehensive theory on the origin of flowers must account for all the

major morphological evolutionary traits of the flower instead of focusing on the evolution of one organ (Frohlich, 2006). More robust insights would be obtained from more integrative models that consider multiple traits in both extant plants and fossil records, high-quality taxonomic data, molecular mechanisms of character evolution, and information in databases cataloguing georeferenced occurrences.

AUTHOR CONTRIBUTIONS

YL planned and designed the research, performed experiments, collected images and drawn the illustration, and wrote the article; WD performed experiments and complemented the writing; SW complemented the writing; X-FW supervised the experiments and complemented the writing.

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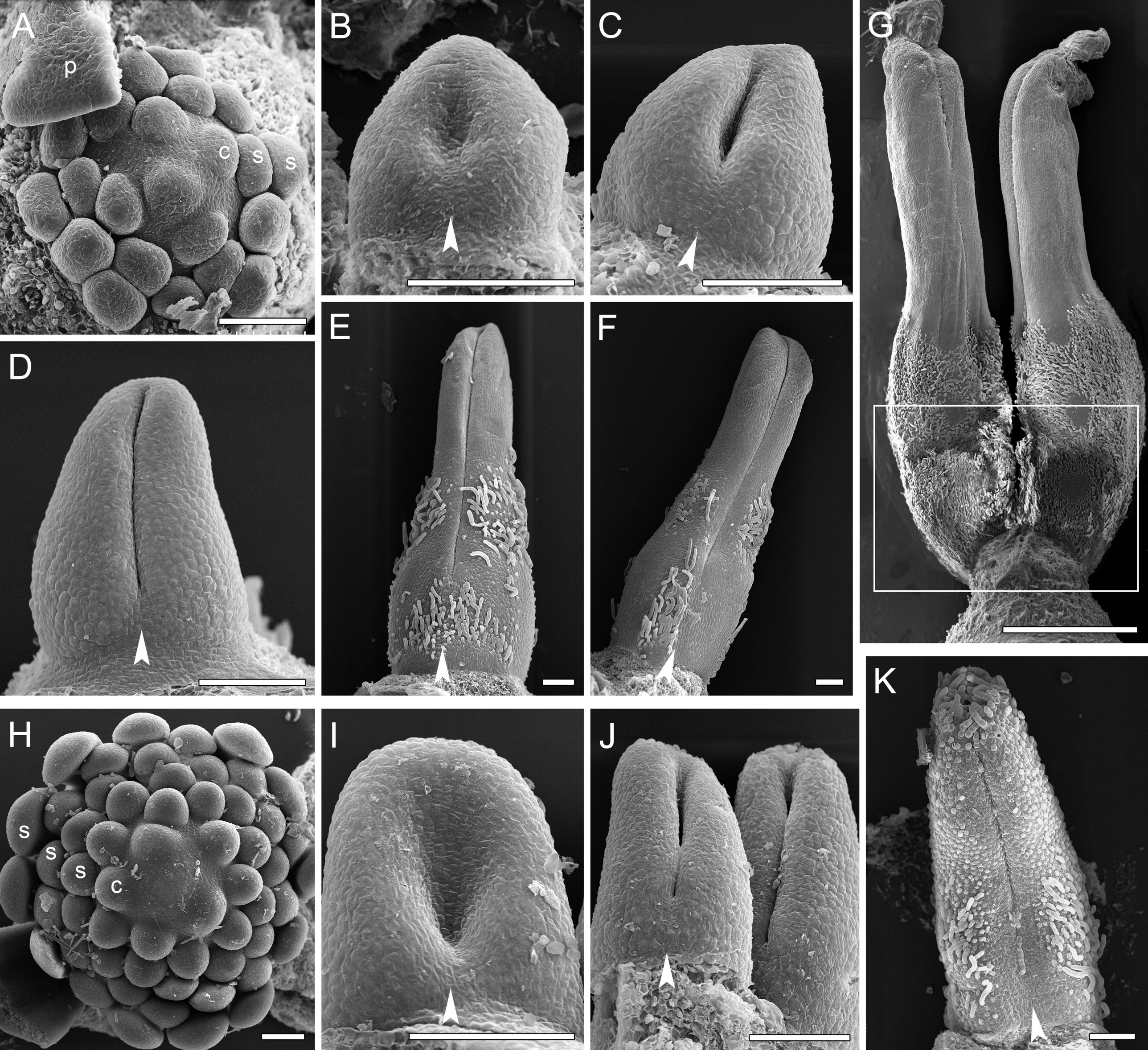
FIGURE 1. Scanning Electron Micrograph of *Anaxagorea* carpel. **(A–G)** *Anaxagorea luzonensis.* **(A)** Carpel primordia. **(B–C)** Emergence of the basal stipe. **(D)** Incomplete stipe closure. **(E–F)** Carpel thickening and stigma formation. **(G)** Carpel stipes at anthesis (box). **(H–K)** *Anaxagorea javanica* shows similar carpel developmental features correlated with changes depicted in **(A, C, D, F)**. Carpel stipes are marked by arrows. Abbreviations: c, carpel; s, stamen; p, perianth. Scale bars: **(A–F, H–K)** = 100 μm; **(G)** = 1 mm.

FIGURE 2. Paraffin sections of carpels stained with Safranin O and Fast Green. **(A–D)** Anthetic carpels of *Anaxagorea luzonensis*, from the base upward. **(A)** The ring-arranged collateral bundles (RACBs) at the base of the carpel, ventral sides are marked by arrows. **(B)** Bundle complexes with xylem surrounded by phloem in each carpel (ring marked). **(C)** A short ascidiate zone. **(D)** Ovule bundles separate from the "C"-shaped bundle complexes (CBCs). **(E–H)** Mature carpel of *A. luzonensis*. **(E)** Two sets of RACBs at the level of **(B)**. **(F)** Ventral bundles (vb) and ovule bundles (ob). **(G)** Detailed view of a vb from **(F)**. **(H)** Detailed view of an ob from **(F)**. **(I–M)** Mature carpel of *Anaxagorea javanica*, from the base upward. **(I)** RACBs at the base of the carpel, a distinct collateral bundle marked by a ring. **(J–K)** Bundles rearranged into two sets of RACBs. **(L)** The CBCs with phloem merge. **(M)** The base of the ovule locule. Abbreviations: db, dorsal bundle; vb, ventral bundle; ob, ovule bundle. Scale bars = 200 μm.

FIGURE 3. Drawings of sequential sections of a mature *Anaxagorea* carpel. Vasculature colored with phloem dark green and xylem red; distinct vascular bundles within the vascular complex are outlined. **(A)** Schematic longitudinal section with ventral side on the left. Vertical zones labeled with letters correspond to the transverse sections of panels to the right. **(B–M)** Transverse sections (ventral side up) are described from the base up to the carpel. **(M)** The ring-arranged collateral bundles (RACBs) at the base of the carpel. **(J–L)** Vasculature changes from a set of RACBs at the base of the carpel to two sets of RACBs. **(H–I)** Vasculature changes from each set of RACBs to the "C"-shaped bundle complexes (CBCs). **(E–G)** Ovule bundles are separated from the CBCs, while other bundles are into the carpel wall. **(B–D)** Meshy bundles in the carpel wall. Abbreviations: db, dorsal bundle; vb, ventral bundle; ob, ovule bundle. Scale bars = 500 μm.

FIGURE 4. Homology comparison between *Anaxagorea* carpel vasculature and female reproductive shoots of seed plants. Colors represent homologies. Cordaitales is a Carboniferous–Permian gymnosperm group. On female reproductive shoots of Cordaitales, ovules (yellow) are interspersed with sterile scales (light green), and ovular axis bearing bracteoles (yellow). The bracteoles on the ovular axis may aggregate into the ovule and the ovular axis may become shorter. Arrangements of the ovules on the shoot apex (dark green) and along the lengths of the ovular axis are relatively flexible. *Ephedra*, a genus representing gnetophytes, which display female reproductive shoots with subtending bracts (light green), have a pair of ovules (yellow)

at their apical end with the abortive apex of the shoot axis (dark green) lying in between. The axis bearing a pair of ovules and their envelope is called a gynocladal axis.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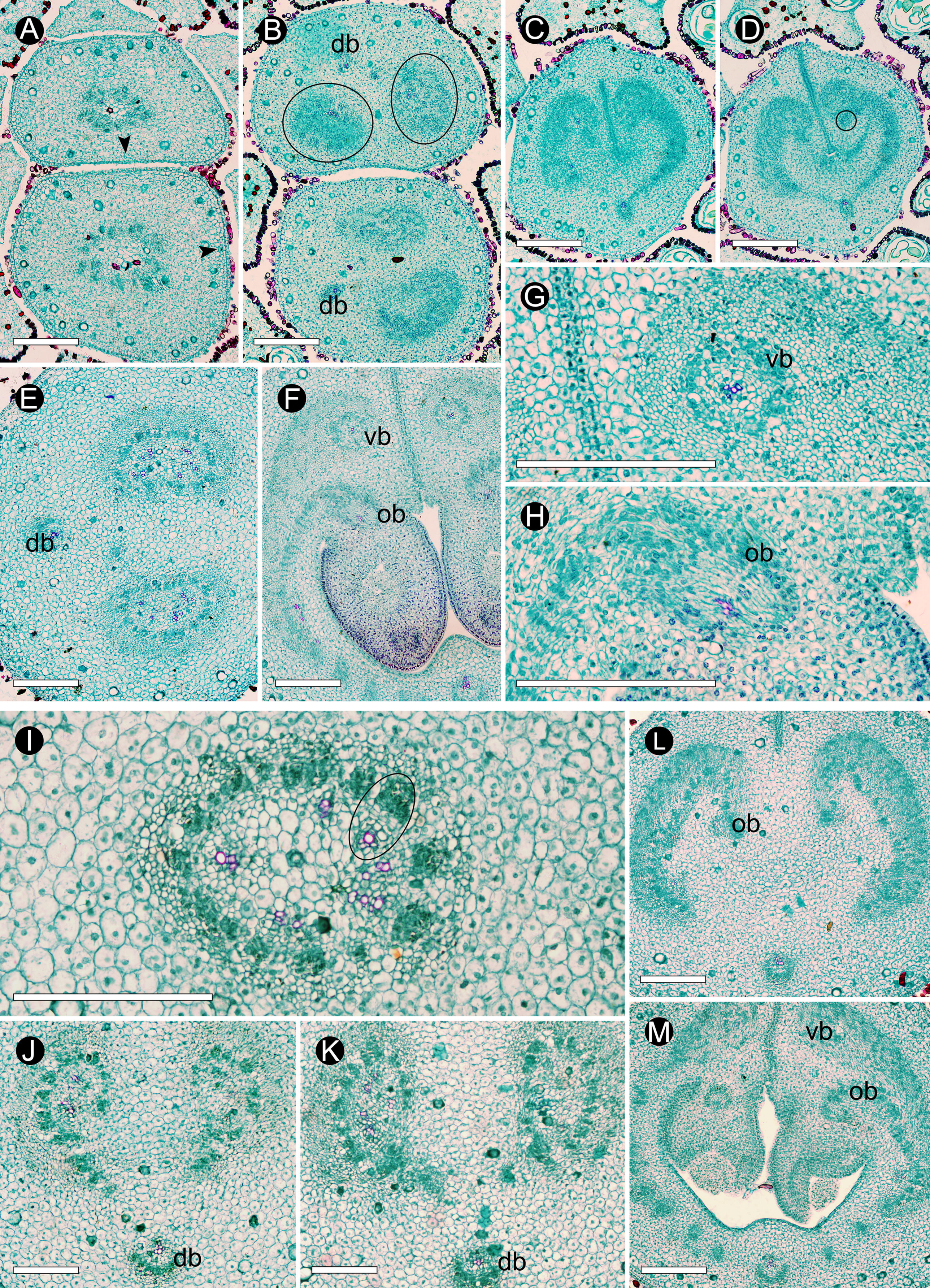
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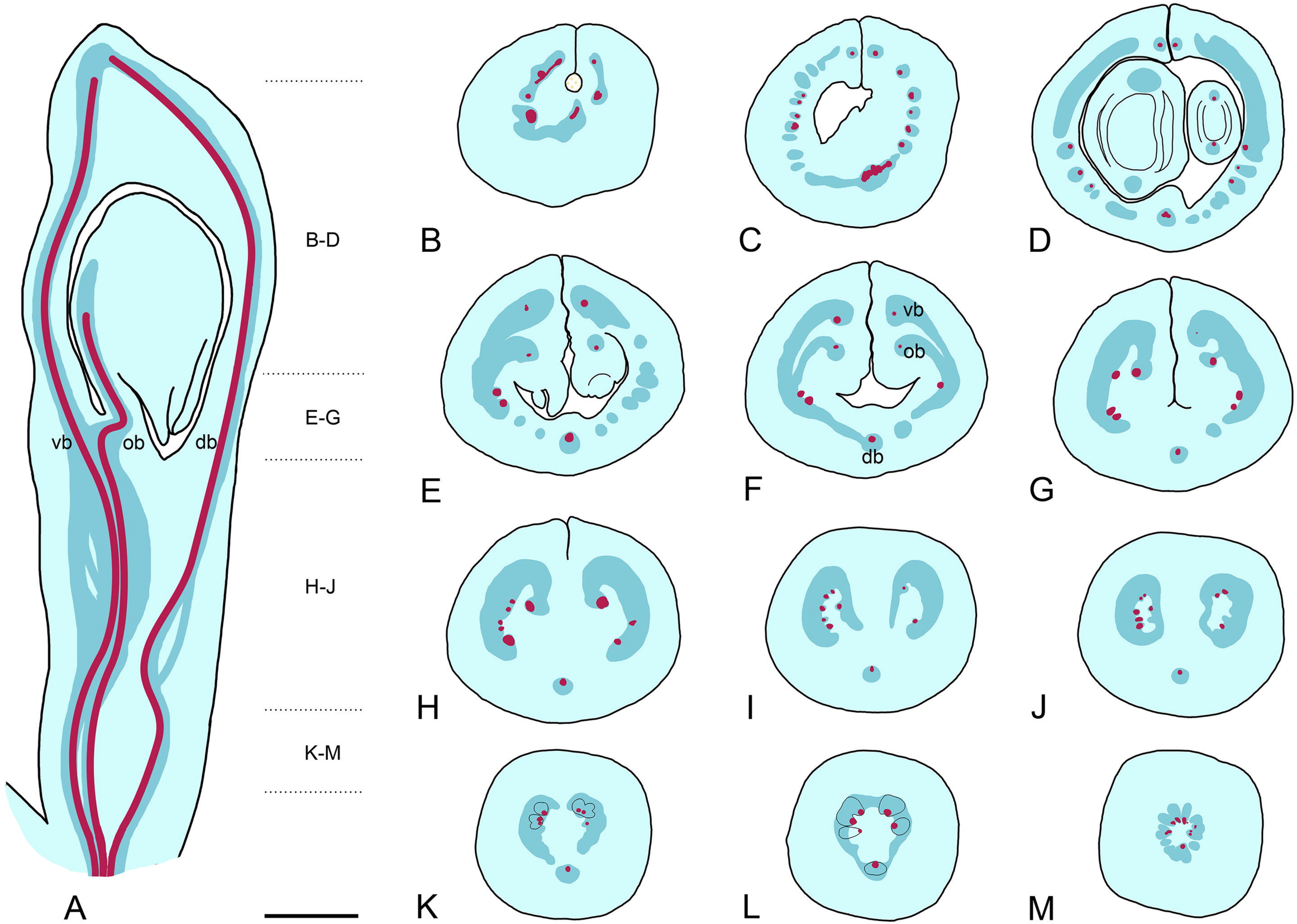
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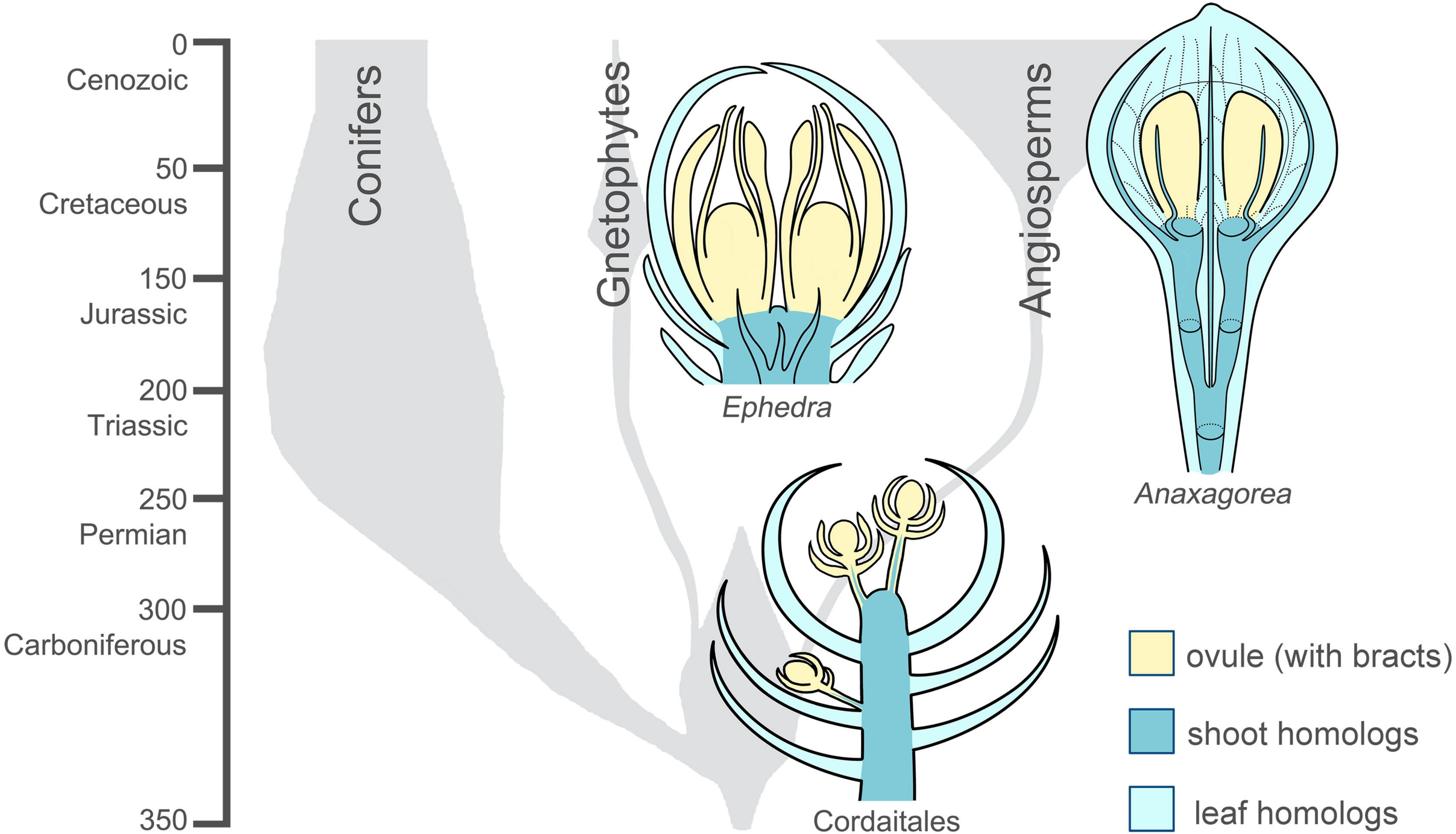
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Cenozoic
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Gnetophytes
Angiosperms
Ephedra
Anaxagorea
Cordaitales
ovule (with bracts)
shoot homologs
leaf homologs