1 **Organogenesis and Vasculature of *Anaxagorea* and its Implications for the**
2 **Integrated Axial-Foliar Origin of Angiosperm Carpel**
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11 Running Title: The Axial-Foliar Origin of Carpel
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Abstract

The carpel is the definitive structure of angiosperms, the origin of carpel is of great significance to the phylogenetic origin of angiosperms. Traditional view was that angiosperm carpels were derived from structures similar to macrosporophylls of pteridosperms or Bennettitales, which bear ovules on the surfaces of foliar organs. In contrast, other views indicate that carpels are originated from the foliar appendage enclosing the ovule-bearing axis. One of the key differences between these two conflicting ideas lies in whether the ovular axis is involved in the evolution of carpel. The aim of this paper is to investigate whether the axial homologs remain in the carpel of extant angiosperms, and thereby to prove the carpel is integrated axial-foliar originated. Two *Anaxagorea* species was used for organogenesis and comparative anatomical study due to its outstanding and unusually long carpel stipe. The continuous change of vascular bundles at carpel stipe in different developmental stages was described in detail. Organogenesis study shows that the carpel stipe of *Anaxagorea* occurs at the early stage of carpel development. Vascular bundles at the base of *Anaxagorea* carpel are a set of discrete ring-arranged collateral bundles (RACBs), which branch out at the upper portion into two sets of RACBs below each ovule. The ring-arranged collateral bundles indicates a clear morphological evidence for the existence of the axial homologs in the carpel and thus support the idea that carpels originated from the integration of the ovular axis and foliar parts. This finding may also promote reconsiderations of the phylogenetic relationship between angiosperms, gnetophytes and Cordaitales.

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

INTRODUCTION

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

The history of exploring the origin of carpel has been tortuous. Competing theories of flower origins provide differing explanations and shaped different 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). Alternatively, the stachyosporous origin theories suggest that the carpel is originated from a compound shoot with the integration of the ovular axis and foliar appendage, the placenta with ovules in the carpel is homologous with a female short-shoot of gymnosperms with distally placed ovules, each with scales forming the outer integument (Stebbins, 1974; Retallack and Dilcher, 1981; Crane, 1985; Doyle and Donoghue, 1986; Nixon *et al.*, 1994; Hickey and Taylor, 1996; Wang, 2010, 2018). The Caytonialean hypothesis holds that the cupule wall of seed ferns provides a homolog for the outer integument of angiosperm ovule and that expansion and folding of the cupule-bearing axis became the progenitor of the carpel (Doyle, 1978, 2006, 2008). And in still another fashion, the carpel has evolved by ectopic expression of ovules on a phyllome (e.g., the Mostly Male theory [Frohlich and Parker, 2000]).

However, since the sister group of the angiosperms is unknown, the origin of carpel is still enigmatic. Progress has been made in the study of morphology and functional genes among angiosperms. The placenta and ovary wall of angiosperms were respectively served by independent vascular bundles (Guo et al., 2013; Liu et al., 2014; Guo et al., 2017; Zhang et al., 2017), and were controlled by different genes that the corresponding to a branch organ and a leaf organ 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 axis and carpel wall (e.g., *Gyrostemon* [Hufford, 1996]; *Illicium* [Zhang et al., 2019];

83   *Phytolacca* [Zhang et al., 2018]). These are necessary clues for the carpel if the
84   placenta and the carpel wall have different evolutionary origins, but more solid
85   evidence is still lacking. According to the above-mentioned hypotheses, it is critical to
86   find clear evidences for the existence of axial homologs in the carpel for the origin of
87   angiosperms. Following the logic that there is no doubt the carpel wall is derived from
88   foliar homologs, as long as the axial homologs in the carpel are determined, the carpel
89   should rather derived from an ovule-bearing axis fused with the leaf-like structure
90   than derived from the megasporophyll.
91
92   To find a more dependable evidence for the existence of axial homologs, the carpel of
93   *Anaxagorea* (Annonaceae) was selected for organogenesis and vascular anatomic
94   examination. Annonaceae are one of the largest families of Magnoliales, which is one
95   of the most important lineages in the early radiation of angiosperms (Sauquet et al.,
96   2003). *Anaxagorea* is phylogenetically the most basal genus in Annonaceae (Doyle
97   and le Thomas, 1996; Doyle et al., 2004; Chatrou et al., 2012; Chatrou et al., 2018),
98   which live in dimly-lit riparian zones in forest slope understory habitats. In this genus,
99   plants have simple leaves that are alternately arranged in two rows along the stems
100   and the flowers usually have whorled phyllotaxis. Gynoecium are apocarpous (free
101   carpels) throughout the life history (Deroin, 1988). Each carpel of *Anaxagorea* has a
102   notable long stipe (Endress and Armstrong, 2011), which is one of the important
103   characteristics of the genus. Our investigation is based on the considerations that
104   morphological features shared by *Anaxagorea* are relatively primitive in angiosperms,
105   and the presence of the notable carpel stipe makes it possible to determine whether
106   there are "axial homologs" in the carpel through tissue sectioning.
107
108   **MATERIALS AND METHODS**
109   **Study Species and Sites**
110   Flower samples of *Anaxagorea luzonensis* and *Anaxagorea javanica* were collected in
111   July 2017 at the Diaoluo Mountain (Hainan, China) and Xishuangbanna Tropical
112   Botanical Garden (Yunnan, China), respectively. The former usually has two to four
113   carpels, whereas the latter has a multicarpellate gynoecium.
114
115   **SEM and Paraffin Slices**
116   Flower samples were collected at different floral stages (from early bud to young
117   fruit). Gynoeciums were isolated from the other parts of the flower and preserved in
118   formalin–acetic acid–70% alcohol (5:5:90, v/v). The fixed material was dehydrated
119   through a series of alcohol solutions ranging from 50% to 100%. To delineate the
120   structure and development of carpel, some carpels were removed from the
121   gynoeciums and passed through an iso-pentanol acetate series (SCR, Shanghai,
122   China), and then critically point-dried, sputter-coated with gold, and were observed
123   and photographed using a scanning electron microscope (Tescan VEGA-3-LMU,
124   Brno, Czech). To illustrate the vasculature of carpel, some carpels were embedded in
125   paraffin, sectioned serially into sections of 10- to 12-µm thickness, and then stained
126   with Safranin O and Fast Green. Complete transverse and longitudinal series were
127   examined and photographed under a light microscope (Olympus BX-43-U, Tokyo,

128   Japan) and bright field optics.

129

130   **RESULTS**

131   **Carpel Organogenesis**

132   In *A. luzonensis* and *A. javanica*, carpels are free from each other completely (**Figures
133   1G, 2A**). Each carpel consists of a plicate zone (**Figures 1G, 3B–H**), a very short
134   ascidiate zone (**Figure 2C**), and a conspicuous long stipe (**Figures 1G, 3I–M**). Carpel
135   primordia are approximately hemispherically initiated (**Figure 1A**). Carpel stipe
136   forms early at the basal part in the early stage of carpel development (**Figures 1C, J**),
137   remains elongate, takes up roughly a quarter length of the carpel at anthesis (**Figure
138   1G**), and continues to elongate during the fruit-stage. Continuous growth of the flanks
139   on the ventral side of the young carpel raises the early closure. The closure does not
140   extend to the bottom of the carpel (**Figure 1D**). Subsequently, the dorsal part of each
141   carpel notably thickens and the stigma forms (**Figures 1E, F**). At anthesis, carpels are
142   widest at the basal part, with an arch on abaxial side (**Figure 1G**). Each carpel has
143   two lateral ovules with the placentae at the base of the ovary (**Figures 2F, M, 3A**).

144

145   **Carpel Vasculature**

146   Main vascular bundles of *Anaxagorea* carpel are distinct as a dorsal bundle, a pair of
147   ventral bundles, a pair of ovule bundles, and several lateral bundles (**Figures 2F, M,
148   3A, F**). Vascular system here are described from the base up to the carpel for easier
149   comprehension of vascular transformation according to the schematic (**Figure 3**). At
150   the base of each carpel stipe, discrete collateral (a collateral bundle has adaxial xylem
151   and abaxial phloem) bundles form a ring (hereinafter abbreviated as ring-arranged
152   collateral bundles, RACBs) around the pith-like cell groups (**Figures 2A, I, 3M**). At
153   the slightly upper part of the carpel stipe, those discrete bundles tend to join adjacent
154   bundles in phloem (**Figure 3L**), subsequently separate, and finally are gathered into
155   three groups: a dorsal bundle and two sets of lateral bundle complexes (LBCs, **Figure
156   3K**). Each set of LBCs is assembled into an amphicribral (an amphicribral bundle has
157   its xylem surrounded by the phloem) bundle in the young carpel (**Figure 2B**). While
158   in the mature carpel, each set of LBCs is assembled as a set of RACBs, and thus there
159   are two sets of RACBs in one carpel (**Figures 2E, K, 3J**). Below each placenta, each
160   set of RACBs (or amphicribral bundle) is transformed to a set of "C"-shaped bundle
161   complexes (CBCs), from which several collateral bundles separate from each set of
162   CBCs, and merge with each other to form an amphicribral bundle, which is kept
163   separate from other bundles (**Figures 2C, L, 3G–I**). Each set of CBCs allocates an
164   ovule bundle into each ovule and other bundles into the carpel wall, where the ventral
165   bundles are also separated (**Figures 2D, F, M, 3E–F**). On the cross section of the
166   ovary locule, both ventral bundles and ovule bundles are amphicribral (**Figures 2G,
167   H**). In the carpel wall, lateral bundles present as pinnate arranged collateral bundles,
168   which are connected to form meshy complexes by poorly-differentiated strands
169   (**Figures 2M, 3D, E**). The dorsal bundle is also connected with lateral bundles by
170   poorly-differentiated strands (**Figures 2D, 3F**). Upon the ovule locule, bundles in the
171   carpel wall join each other with xylem and phloem and finally diminish (**Figures 3B,**

5

172   **C**).

173

174   **DISCUSSION**

175   This study firstly investigated the organogenesis and the changes of vascular bundle in
176   different development stages of the carpel stipe of *Anaxagorea*. The special
177   arrangement of vascular bundles in the carpel stipe of *Anaxagorea* suggests that there
178   are homologous structures of ovule axis exist in the carpels of modern angiosperms
179   and constitutes an essential step to understand how the carpels evolved from female
180   reproductive secondary shoot and the sterile bract.

181

182   It has been suggested that peltate carpels is plesiomorphic in Annonaceae (Deroin,
183   1988; Igersheim and Endress, 1997; Surveswaran et al., 2010; Couvreur et al., 2011).
184   Carpels of *Anaxagorea* has been reported to have a short ascidiate zone in some
185   studies (Deroin, 1988), while are completely plicate in others (Endress and Armstrong,
186   2011). In the present study, organogenesis shows that the carpel stipe of *A. luzonensis*
187   and *A. javanica* occurs at the early stage of carpel development and continues to
188   elongate with the development of the carpel. It is different from the mericarp stipes of
189   other Annonaceae plants, the latter of which has the ovuliferous upper ovary parts are
190   built firstly, then sterile lower stipes evolve divergent latter (e.g., *Cananga, Uvari,*
191   *Xylopia* [Deroin, 1999]). In flower stage, the ventral slit may continue for some
192   distance along the ovule locule and terminated very close to the base of ovule locule.
193   These variants of may suggest the transformation from peltate carpels to plicate
194   carpels. The carpel stipe in *Anaxagorea* provides a buffer space for the drastic
195   changes in structure of the vascular bundle at the base of carpel, whereas in most
196   angiosperms with apocarpy, the base of the carpel is very close to the placenta and the
197   vascular bundle in this part tends to be simplified (e.g., *Michelia* [Tucker, 1961];
198   *Sagittaria* [Kaul, 1967]; *Drimys* [Tucker, 1975]; *Illicium* [Robertson and Tucker,
199   1979]; *Brasenia* [Endress, 2005]).

200

201   In Annonaceae, it has been recognized that the distal parts of the cortical vascular
202   system are used up in the formation of carpel traces, three to each carpel (Eames,
203   1961). However, in *A. luzonensis* and *A. javanica*, the number of vascular bundles
204   entering the carpel is far more than three, and arranged in a radiosymmetric pattern.
205   Radiosymmetric vasculature possessed universality in the stems of vascular plants
206   (Metcalfe and Chalk, 1979; Evert, 2006; Beck, 2010; McKown and Dengler, 2010;
207   Evert and Eichhorn, 2011). On the cross section of the stem in most angiosperms
208   other than monocots, discrete collateral bundles form a single ring or a continuous
209   hollow cylinder around the pith (e.g., Caprifoliaceae; Leguminosae; Tiliaceae;
210   Ulmaceae). While the in the leaves, vascular distribution are lateral organized in most
211   seed plants, a largest collateral bundle often extends along the long axis of the leaf as
212   a midvein, from which smaller veins diverge laterally (e.g., Pinaceae; Gnetaceae;
213   Magnoliaceae; Gramineae; Brassicaceae), even in the branching veins in *Ginkgo*, each
214   of the veins is a single collateral bundle. In the cross sections of the carpel of
215   *Anaxagorea*, the RACBs at the basal part acts in accordance with a typical form of the

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216 stem vascular anatomy of most angiosperms. In the distal region, a dorsal bundle
217 separated from the basal RACBs, and the remainder gathered into two sets of
218 amphicribral bundles below each placenta, which are developed into two sets of
219 RACBs in the more mature carpel. This vasculature transition may be an important
220 indication that (1) the basal part of the *Anaxagorea* carpel stipe is an axial homolog in
221 nature, and (2) the carpel is integrated axial-foliar originated from the ovular axis with
222 its bract. The branching of RACBs in each carpel seems to be an evidence for the
223 existence of axial homologs in the carpel because the foliar homologs (lateral organs)
224 cannot form such a topology by coiling transformation since the topological structure
225 of the lateral organized bundles is equivalent to a curve, and the curved surface can
226 only form a one-way tunnel (like macaroni pasta) by coiling. However, the branching
227 RACBs (one set of RACBs branch into two sets of RACBs) in the carpel of
228 *Anaxagorea* are equivalent to a three-way tunnel.

229

230 Development of amphicribral bundles into RACBs was observed in the carpel of *A.*
231 *luzonensis* at different stages. The ovule bundles are also appeared amphicribral,
232 which is separated from the CBCs directly. Amphicribral bundles are frequently seen
233 in small branches of early land plants, in monocots, or in young branches of dicots as
234 simplification and combination of stem bundles (Fahn, 1990). In the carpels of
235 *Magnolia* (Liu et al., 2014) and *Michelia* (Zhang et al., 2017), amphicribral bundles
236 were observed supplying the ovules and derived from cortical amphicribral bundles.
237 Whereas in pistils of *Actinidia* (Guo et al., 2013) and *Dianthus* (Guo et al., 2017),
238 amphicribral bundles were reported in the placenta, distinct from the collateral
239 bundles in the carpel wall. Beyond that, amphicribral bundles seems widespread in the
240 placenta and funiculus of angiosperms (e.g., *Papaver* [Kapoor, 1973], *Psoraleae*
241 [Lersten and Don, 1966], *Drimys* [Tucker, 1975], *Nicotiana* [Dave et al., 1981],
242 *Whytockia* [Wang and Pan, 1998], *Pachysandra* [Von Balthazar and Endress, 2002]).
243 Combined with our above-mentioned findings, this would be a reasonable result since
244 the ovule and placenta in the carpel is originated from the ovular axis.

245

246 Considering the relationship between angiosperms and other seed plants lineages has
247 been a lasting challenge. Taylor (1991) once set a goal for research on the origin of
248 angiosperms, that is, correlation between carpel in angiosperms and reproductive
249 structure in other seed plants. The branching RACBs of *Anaxagorea* carpels implies
250 that the carpel might comparable to a compound shoot and provide an understanding
251 of how the bract-bracteole-terminal ovule system in gymnosperms evolved to an
252 angiosperm carpel (**Figure 4**). The Unifying Theory (Wang, 2010, 2018) suggests that
253 angiosperm carpels are derived from the sterile bracts (formed carpel wall) attached to
254 the ovule-bearing secondary shoot (formed ovule/placenta) of Cordaitales, a common
255 ancestor of conifers and gnetophytes (Eames, 1952; Bold, 1973; Hickey and Taylor,
256 1996; Doyle, 2008). Taking Cordaitales as the common ancestor of angiosperms and
257 other gymnosperms may resolve the contradiction between the similar morphological
258 characters and the distant phylogenetic relationship between angiosperms and
259 gnetophytes (Hajibabaei et al., 2006; Wickett et al., 2014; Wan et al., 2018). However,

260 in Doyle's (2008) strict consensus of 18 most parsimonious trees of 364 steps
261 obtained from the analysis with the arrangement of living taxa fixed to a molecular
262 backbone constraint tree, when Cordaitales was putted in the ancestral position of
263 conifers, gnetophytes, and angiosperms, the most parsimonious trees with *Amborella*
264 basal in angiosperms (as in molecular analyses) would be of the anthophyte type,
265 which is conflicts with molecular data. Since those potential ancestors include
266 Cordaitales are fossils which still have many unclear or misinterpreted characters,
267 more evidences were needed from different approaches.
268
269 In conclusion, our results provide a more reliable evidence that the placenta with
270 ovule and the carpel wall has separated evolutionary origin, and thus support the idea
271 that carpels originated from sterile bracts attached to the ovule-bearing shoot.
272 However, this study is limited to only a single genus. We hypothesize that the axial
273 homologs might be a regular presence in angiosperms carpel. However, these
274 homologs may be diverse organized because the vascular bundle in carpel is usually
275 simplified. We hope that more extensive studies, especially that on early diverging
276 angiosperm groups with carpel stipe, such as Cabomboideae, Illicieae, and
277 Schisandreae, in the future will test the validity of this hypothesis. Nature follows the
278 principle that selecting a simple mechanism or module as building blocks for a
279 complex system and then using that module repeatedly in other systems. The
280 presentation of axial homologs in carpel seems the embodiment of this rule.
281 Interestingly, the word "carpel" was defined in the Annonaceae family firstly by
282 Dunal (1817) two hundred years ago, and now new enlightenment was brought to us
283 in this big family once more. Attempting to explain the origin of angiosperms requires
284 integrating morphological and molecular evidence from a wide range of fossil and
285 extant seed plant data. A full theory of flower origins must account for all the major
286 morphological innovations of the flower other than a focus on only one organ
287 innovation (Frohlich, 2006). More robust answers will come from more integrative
288 models with consideration of multiple traits at once from both extant plants and fossil
289 records, high-quality taxonomic data, molecular mechanism of character evolution,
290 and an abundance of databases cataloguing georeferenced occurrences.
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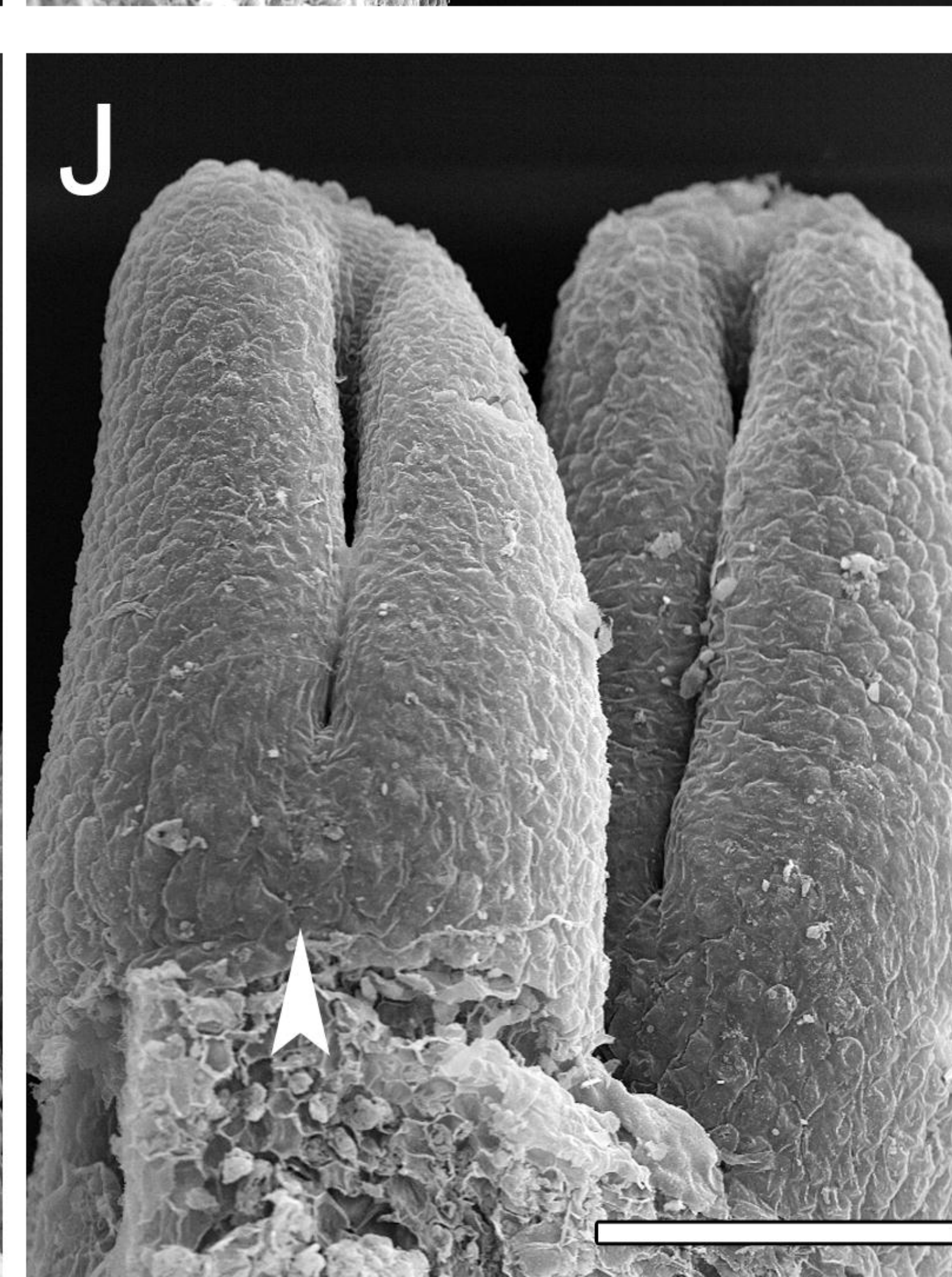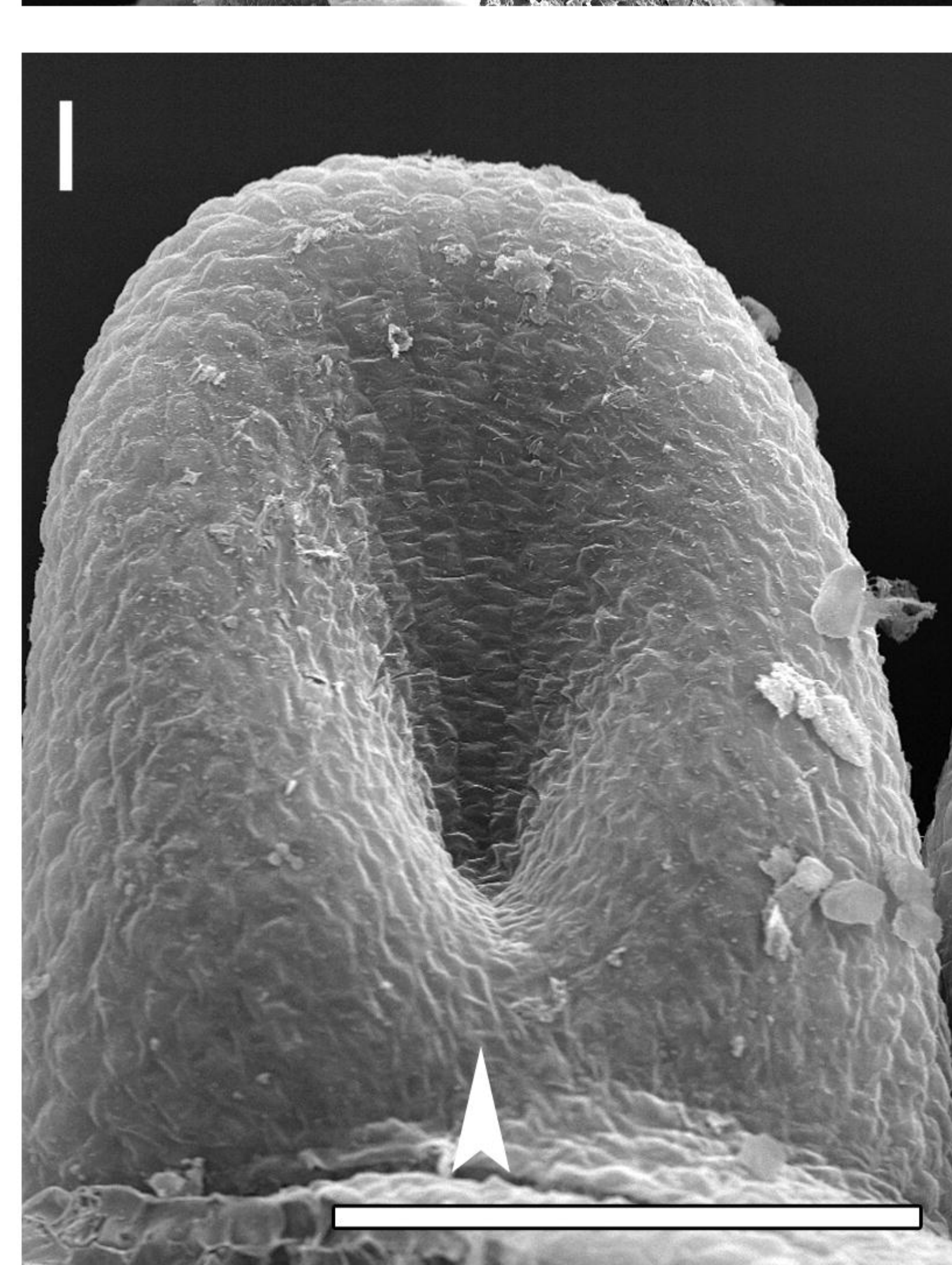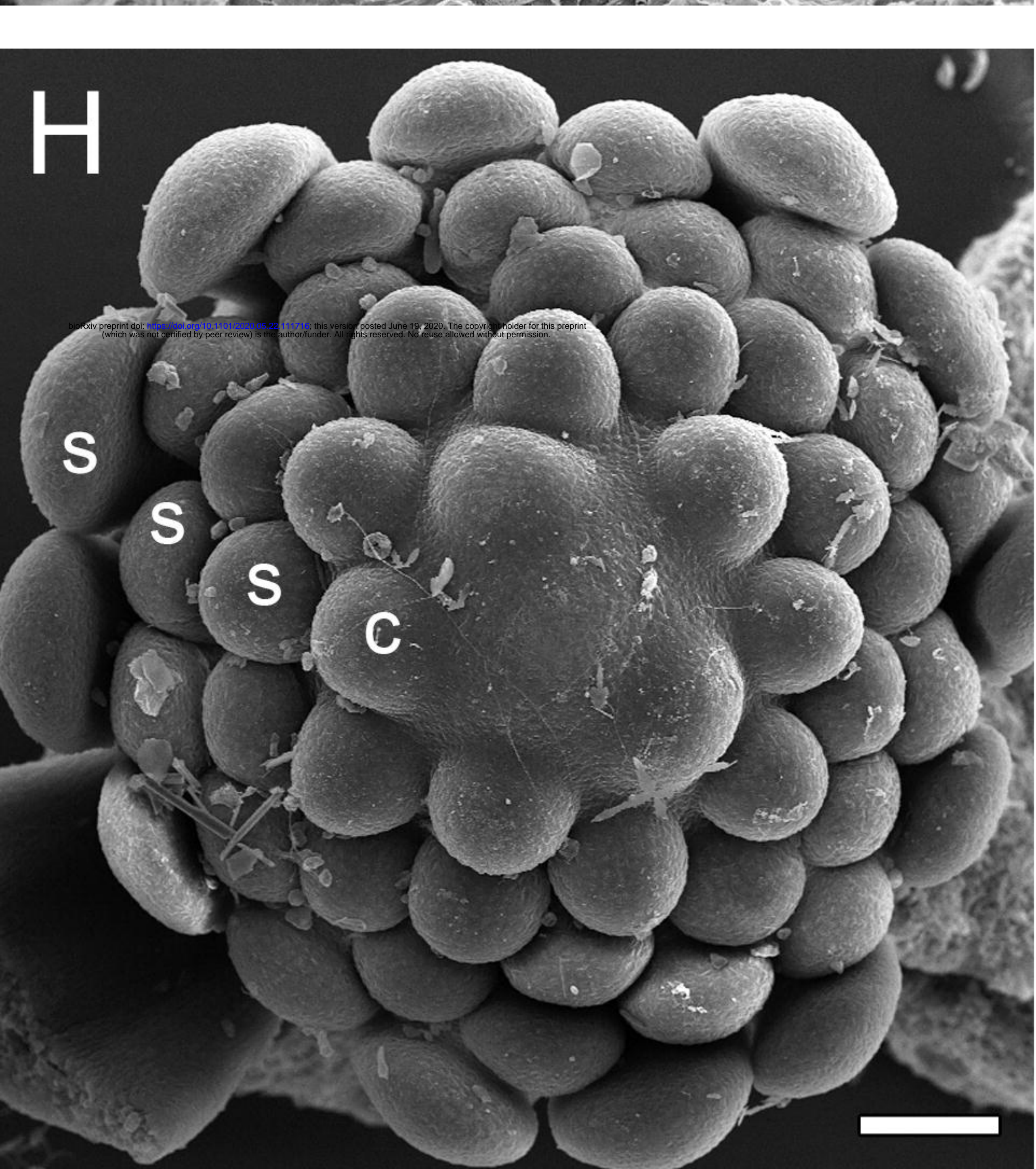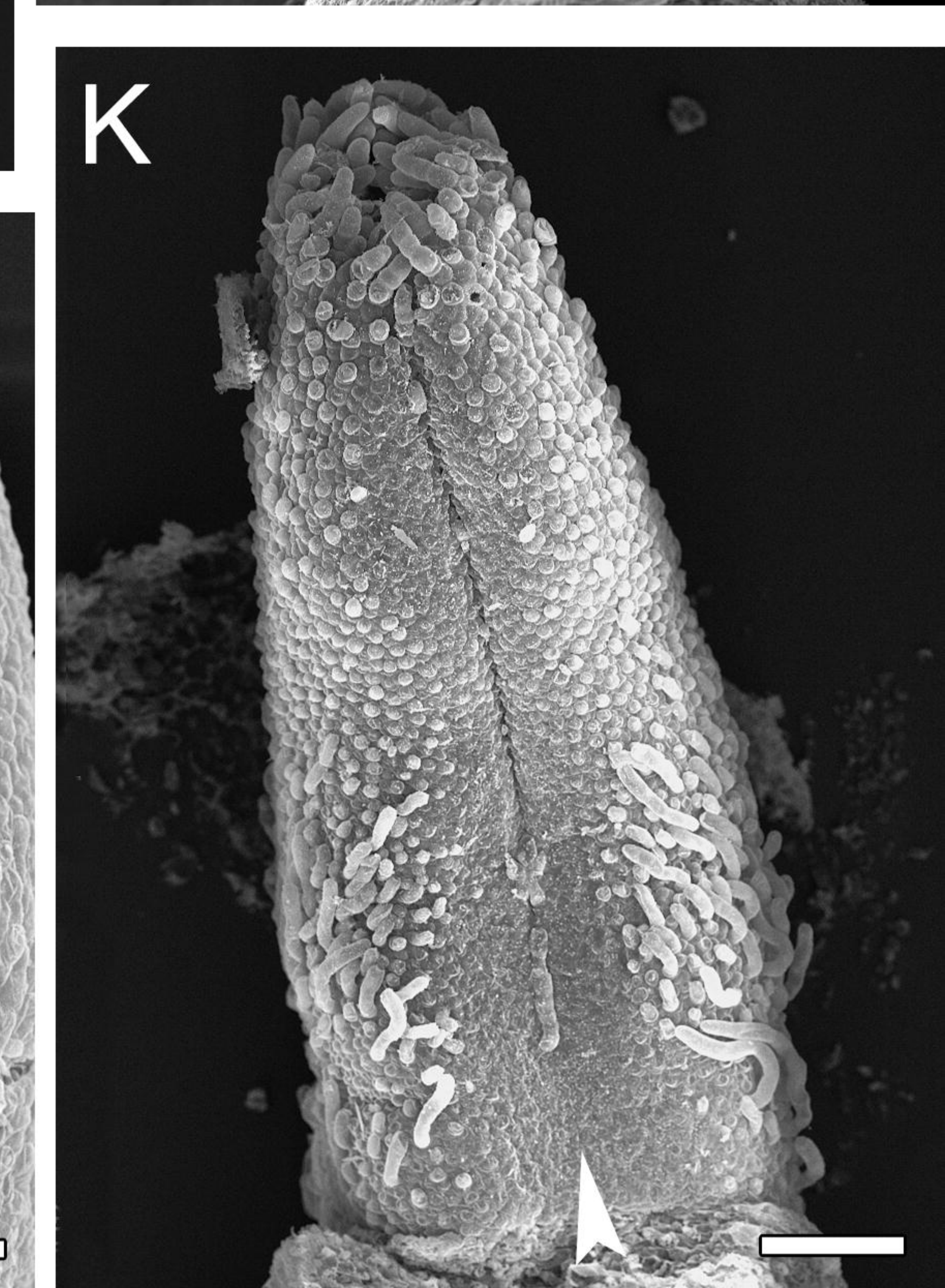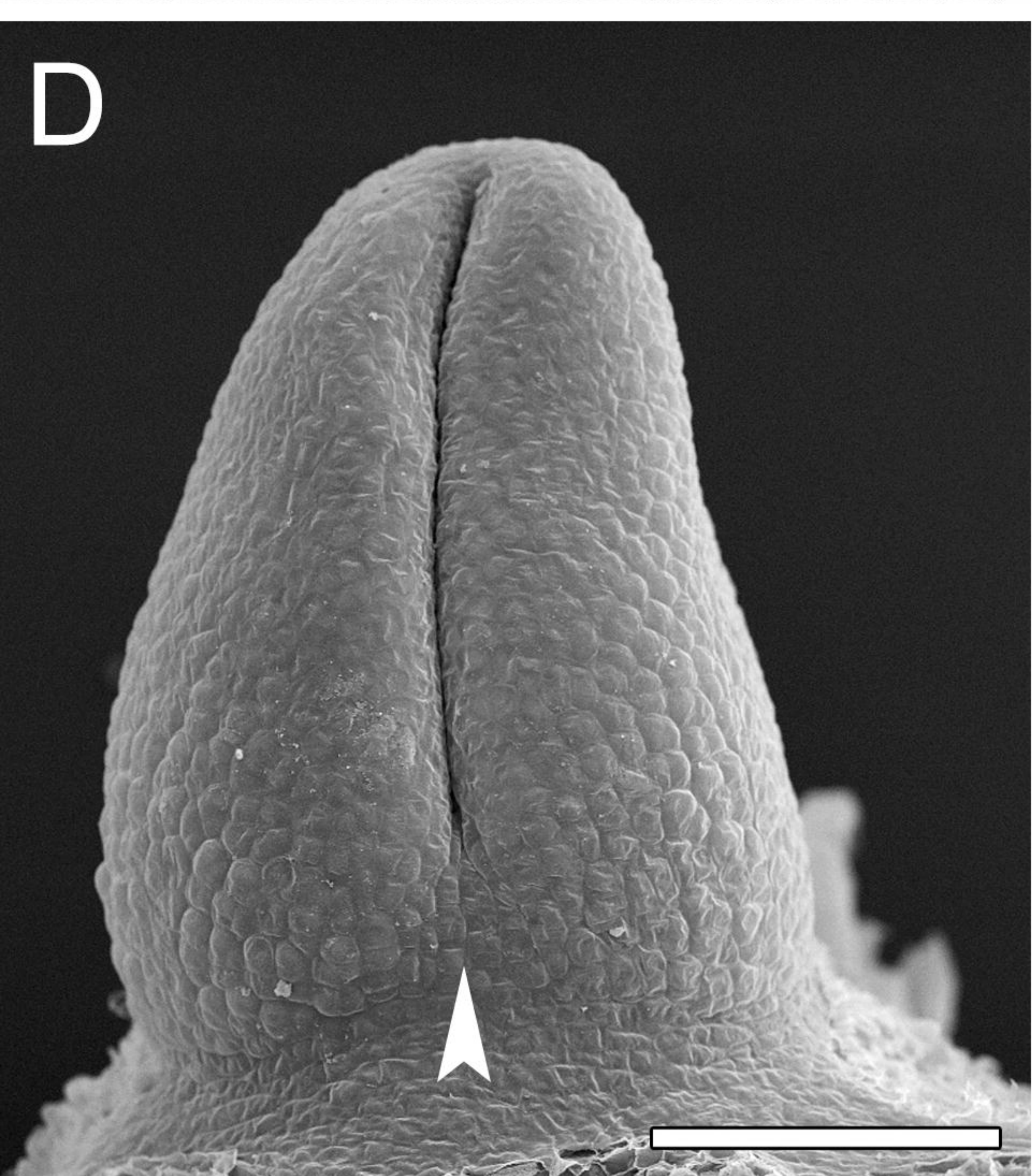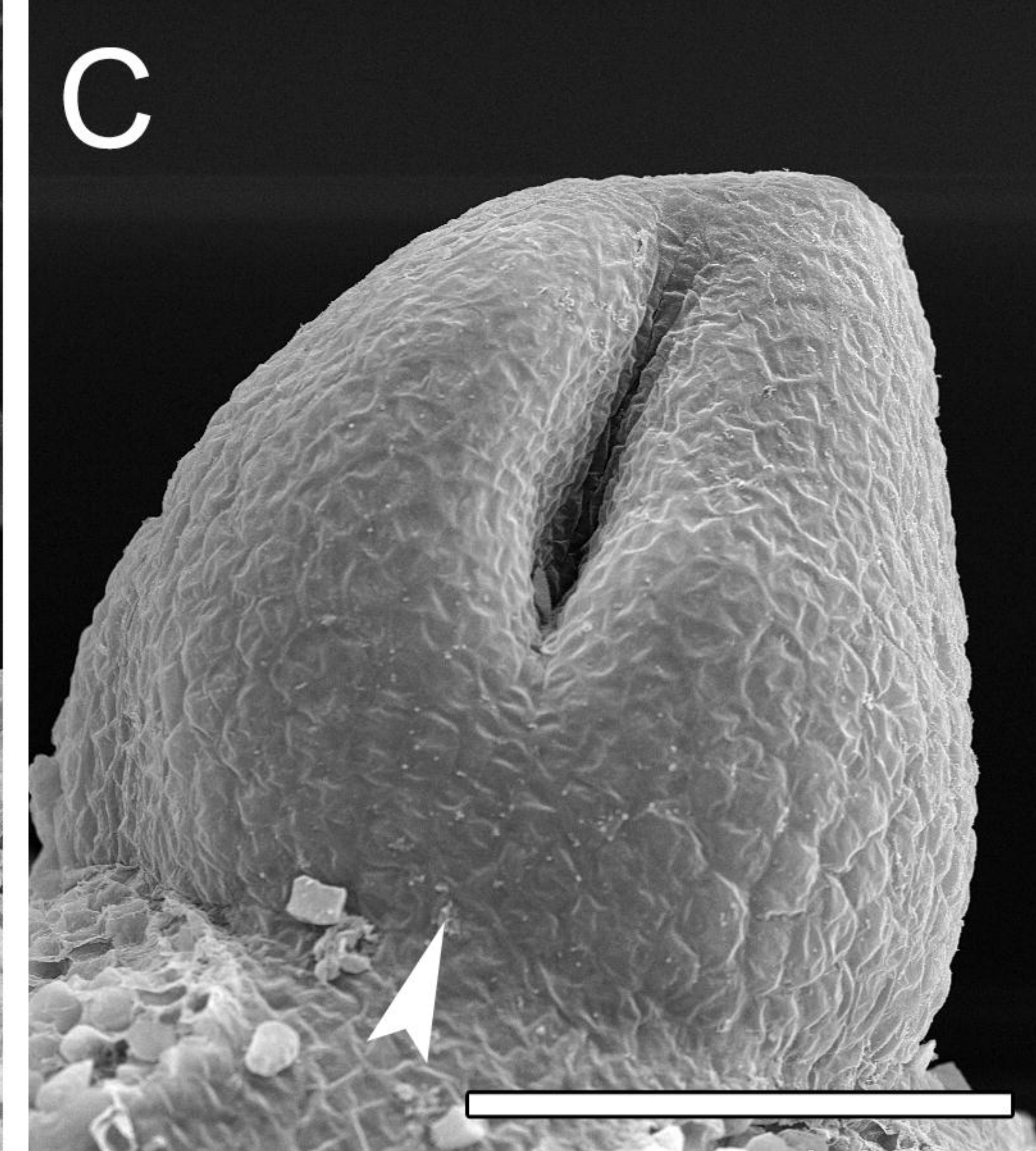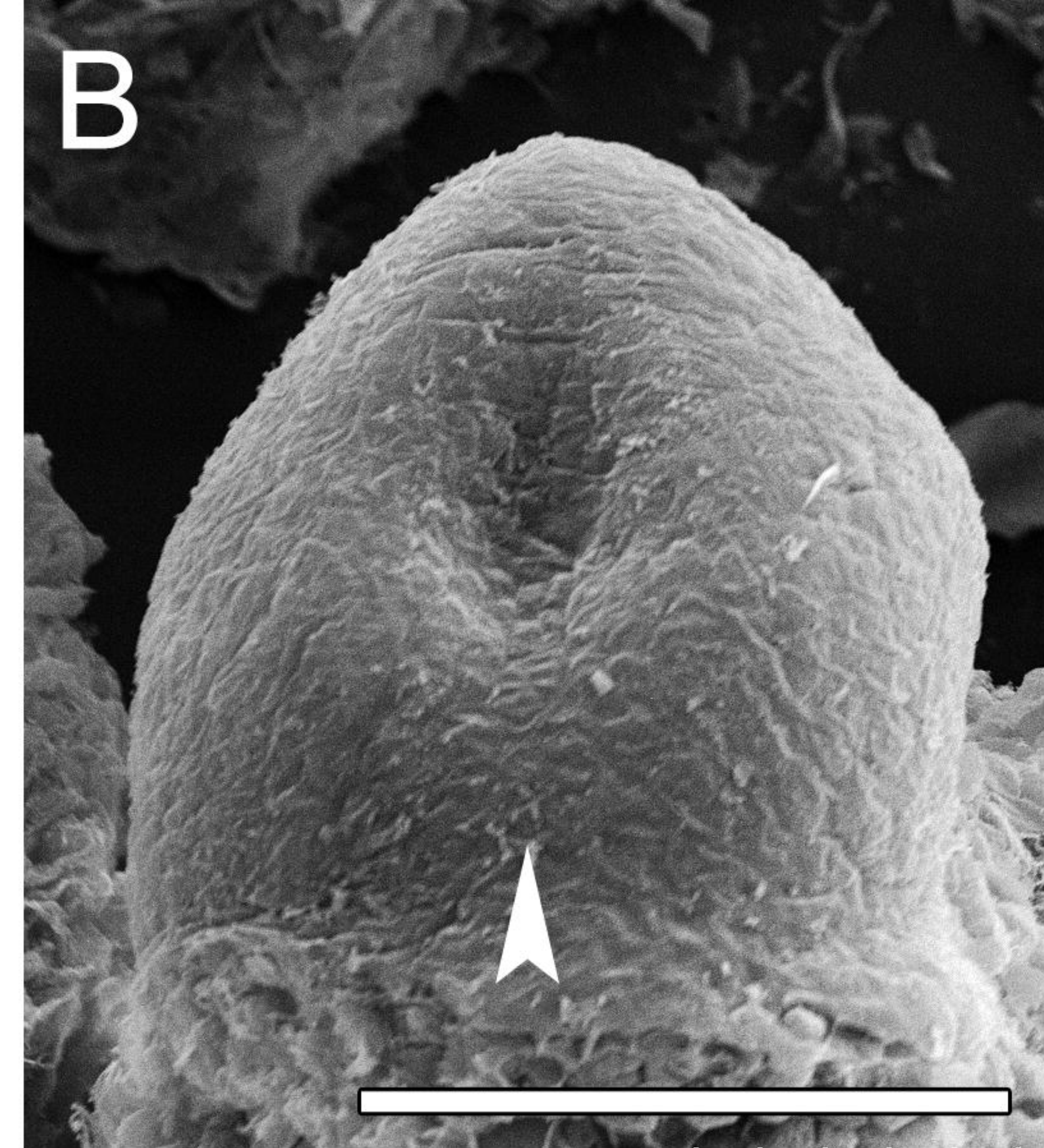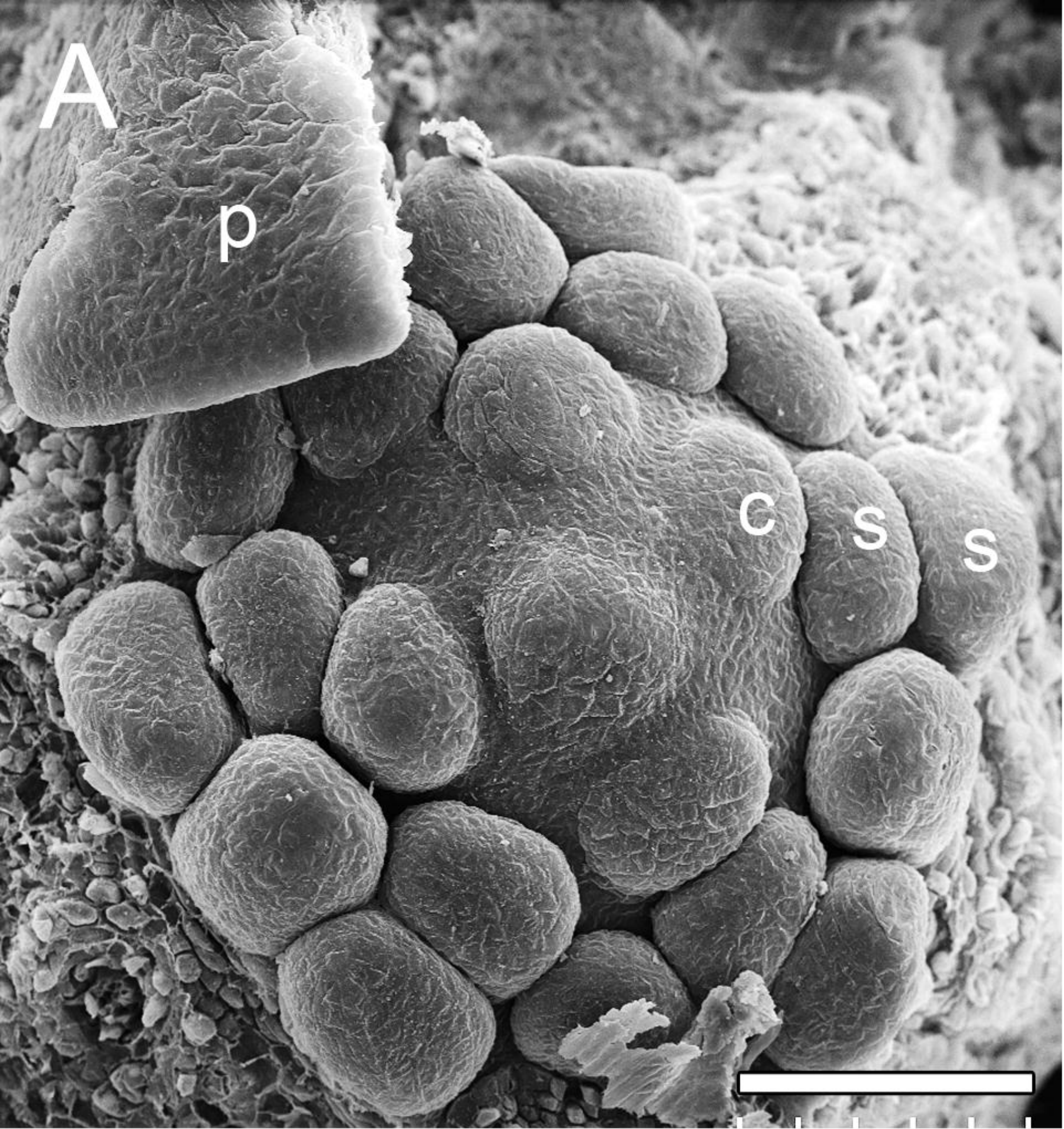
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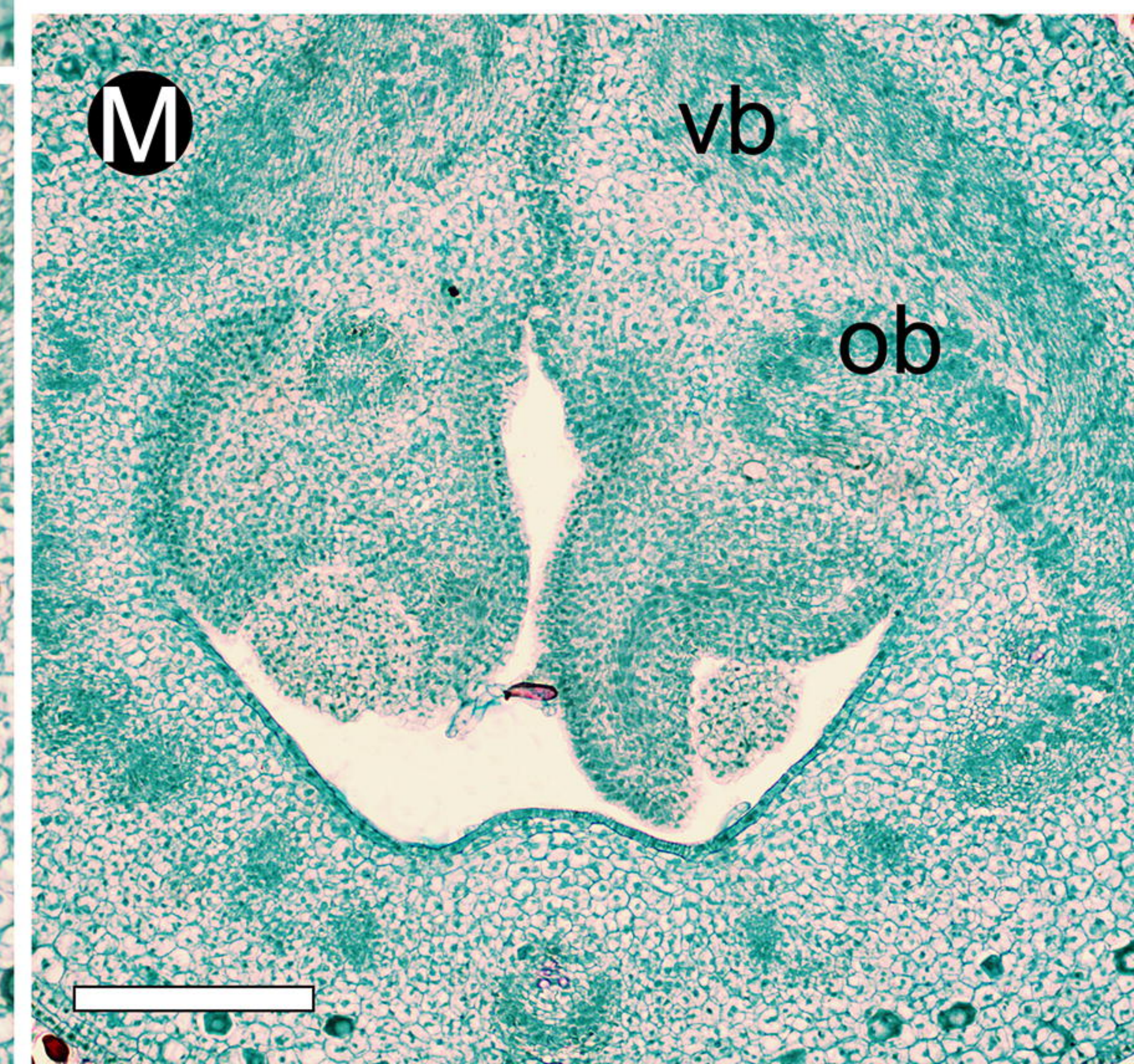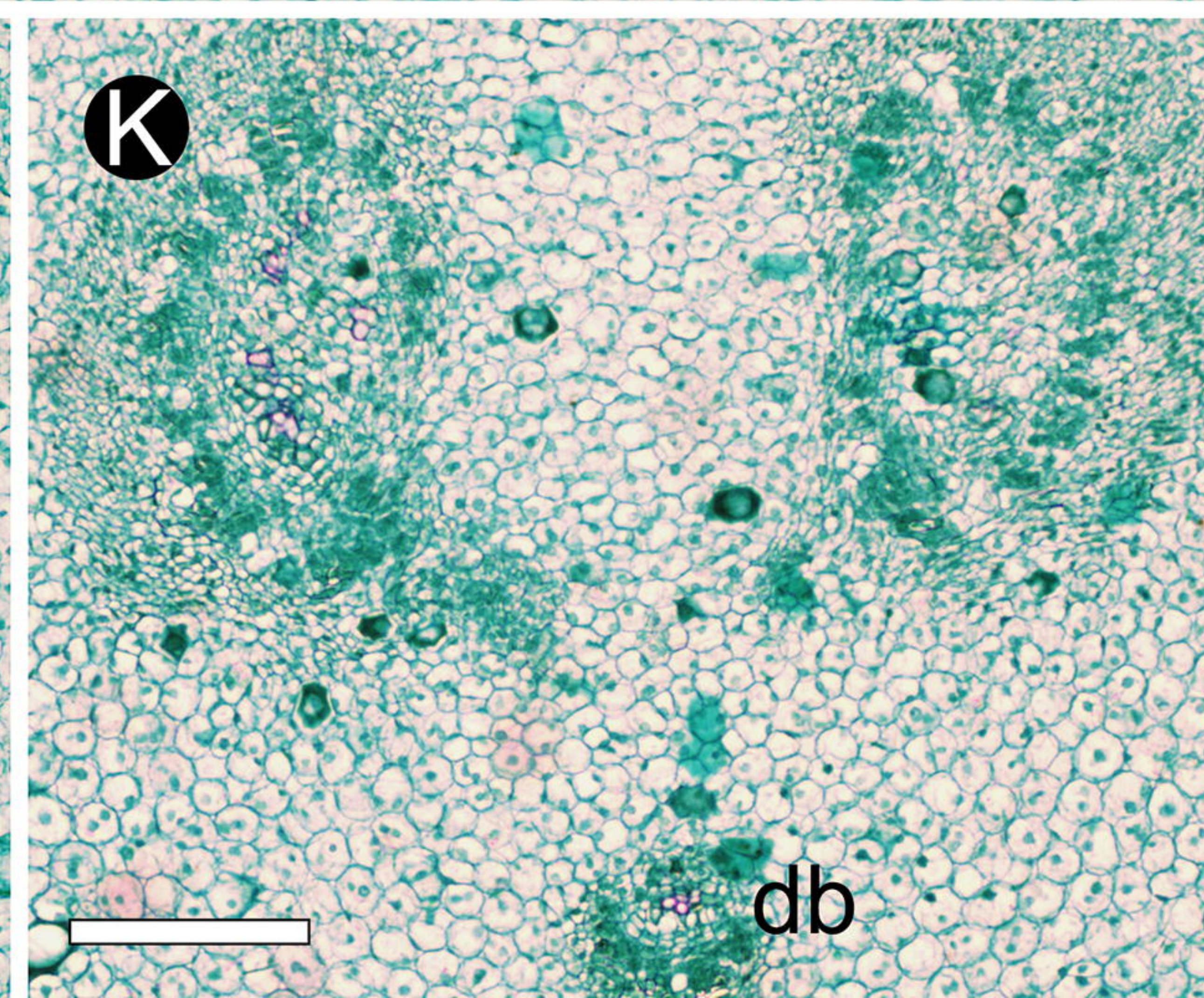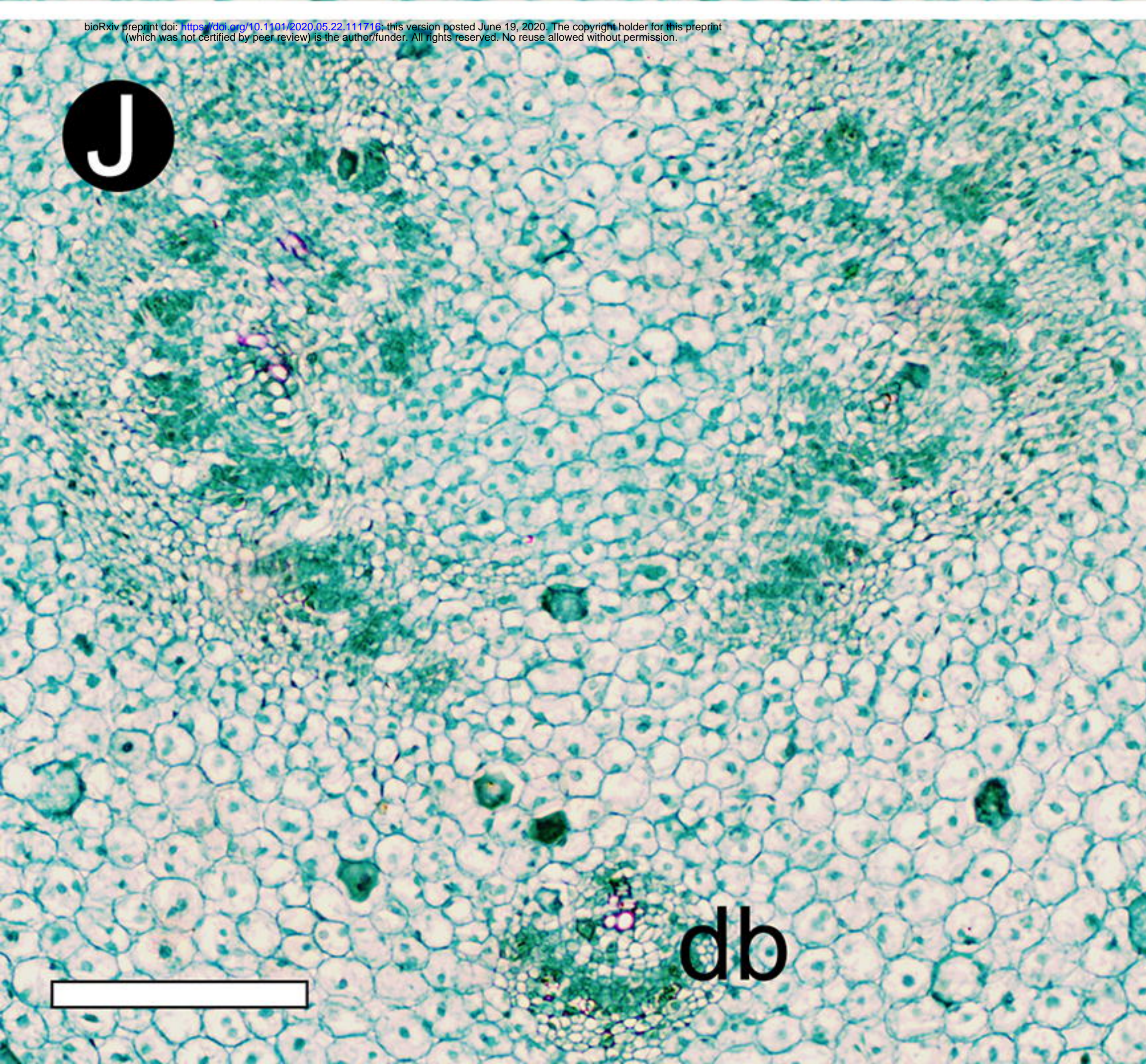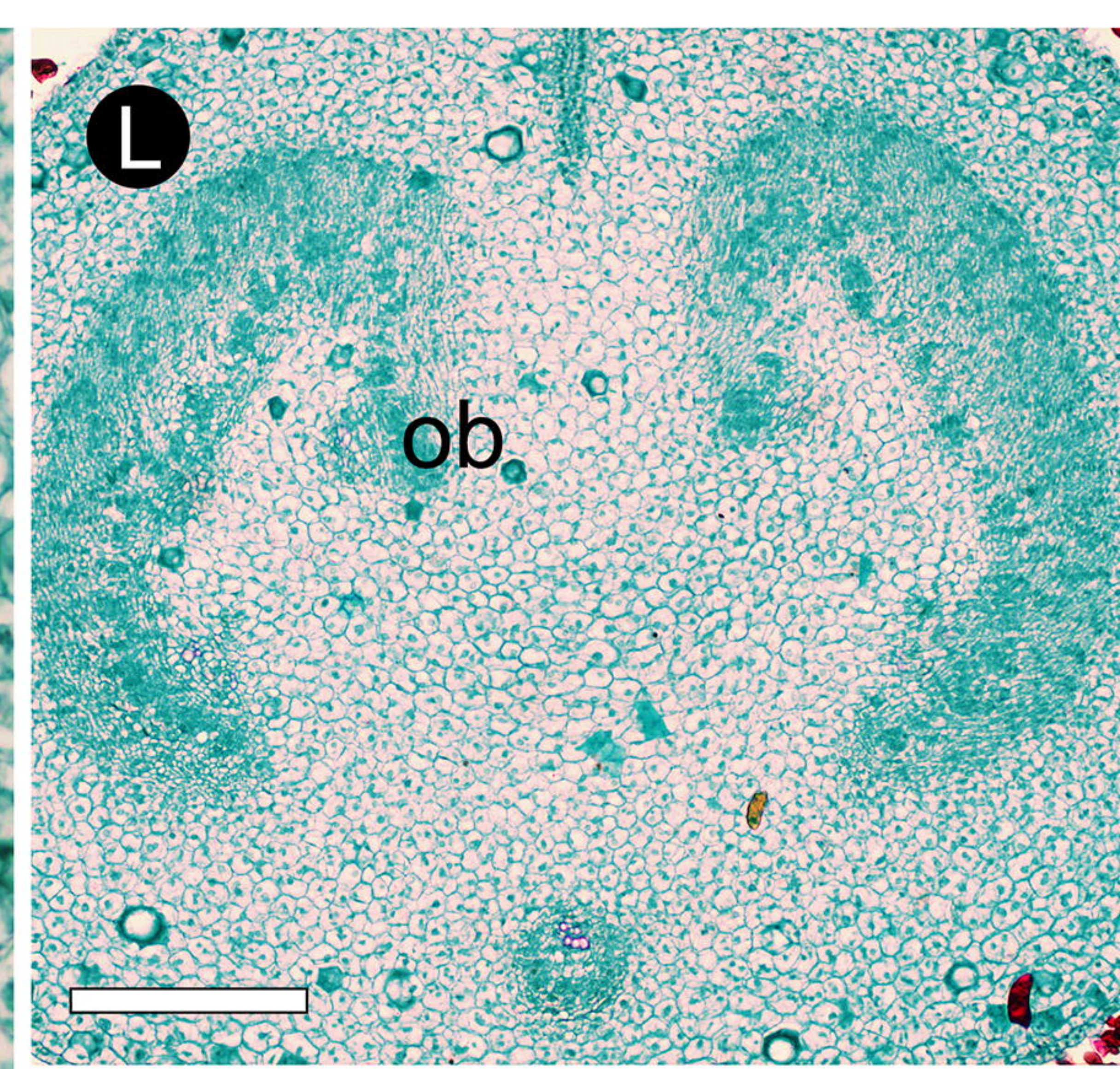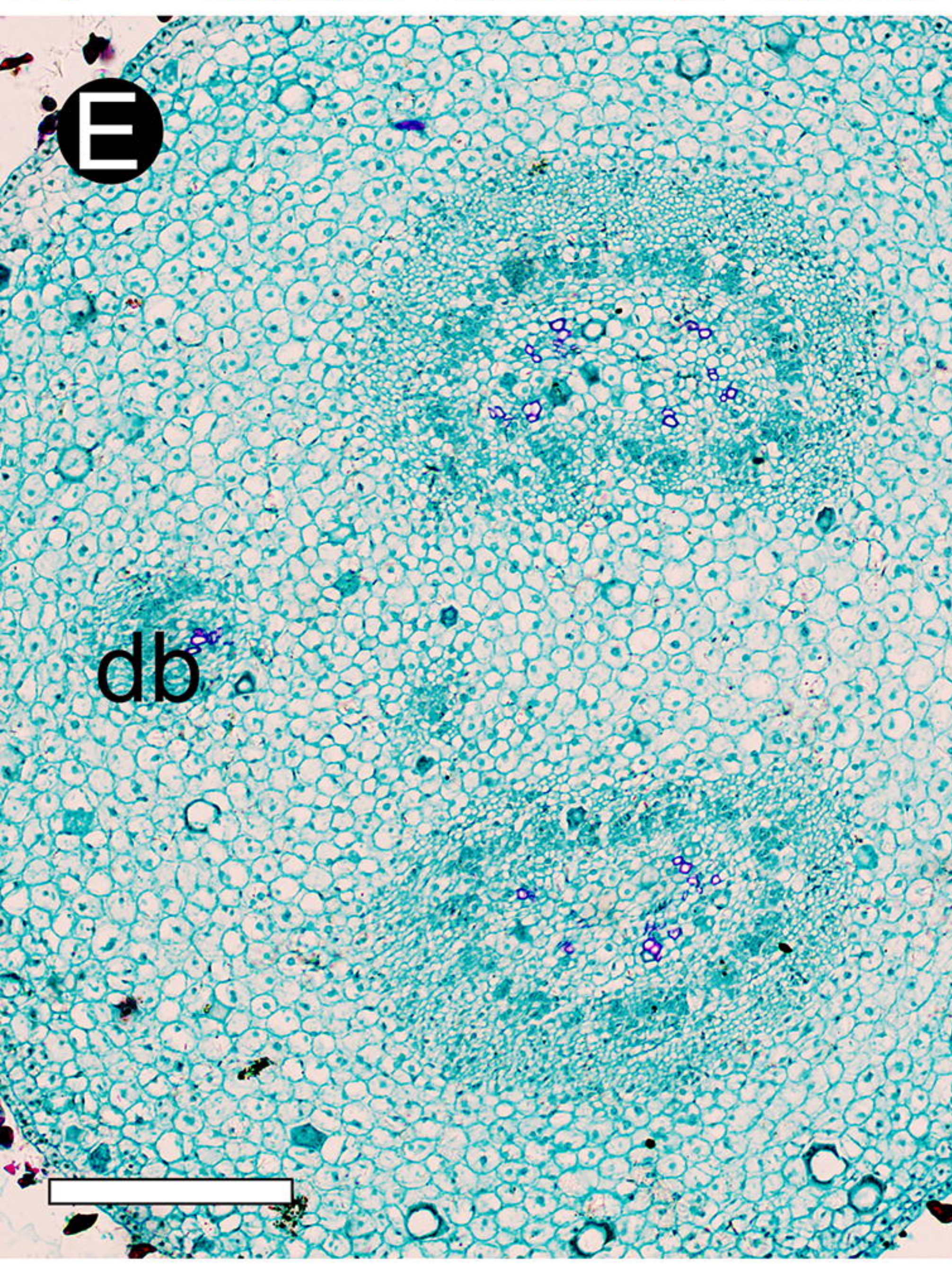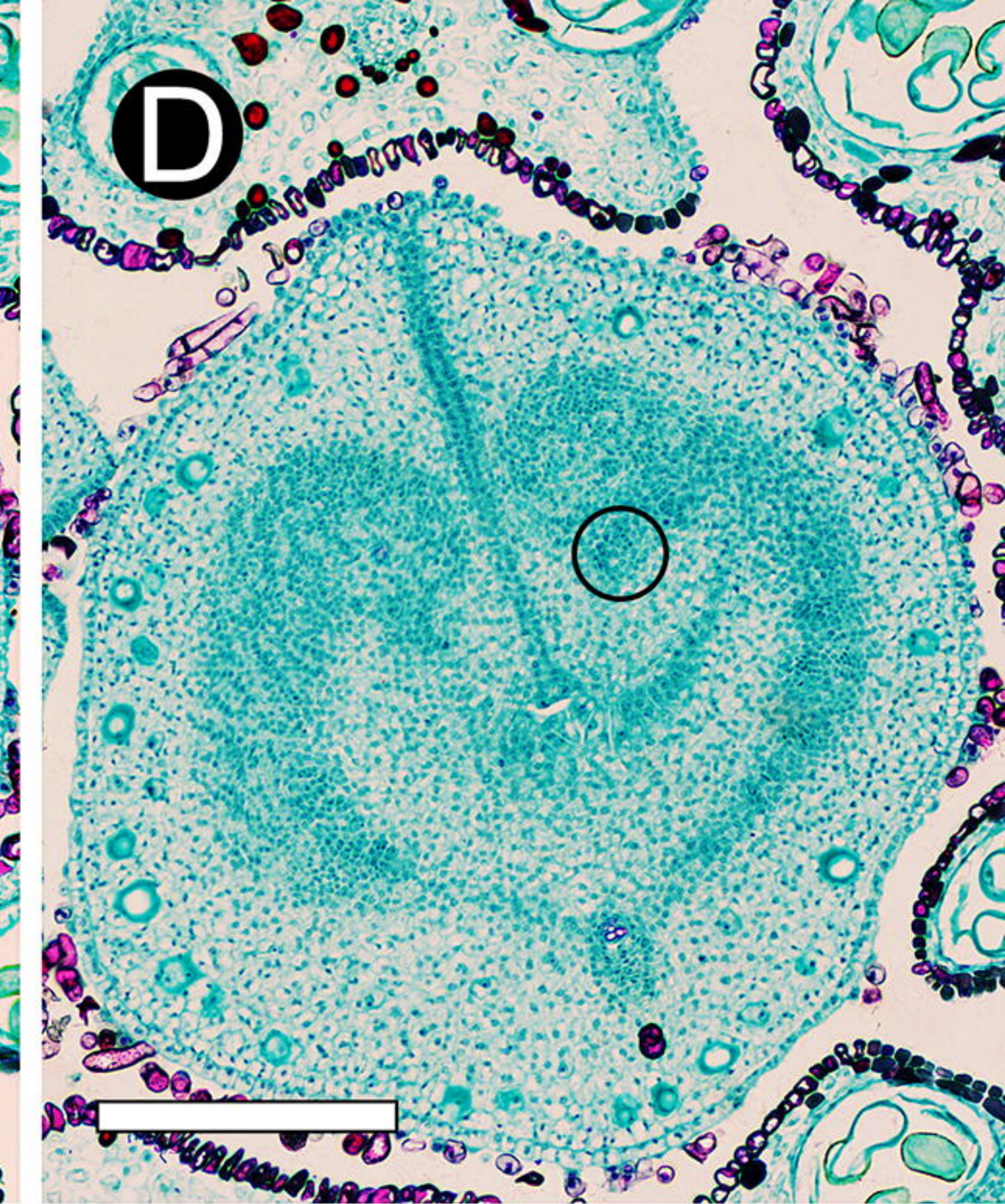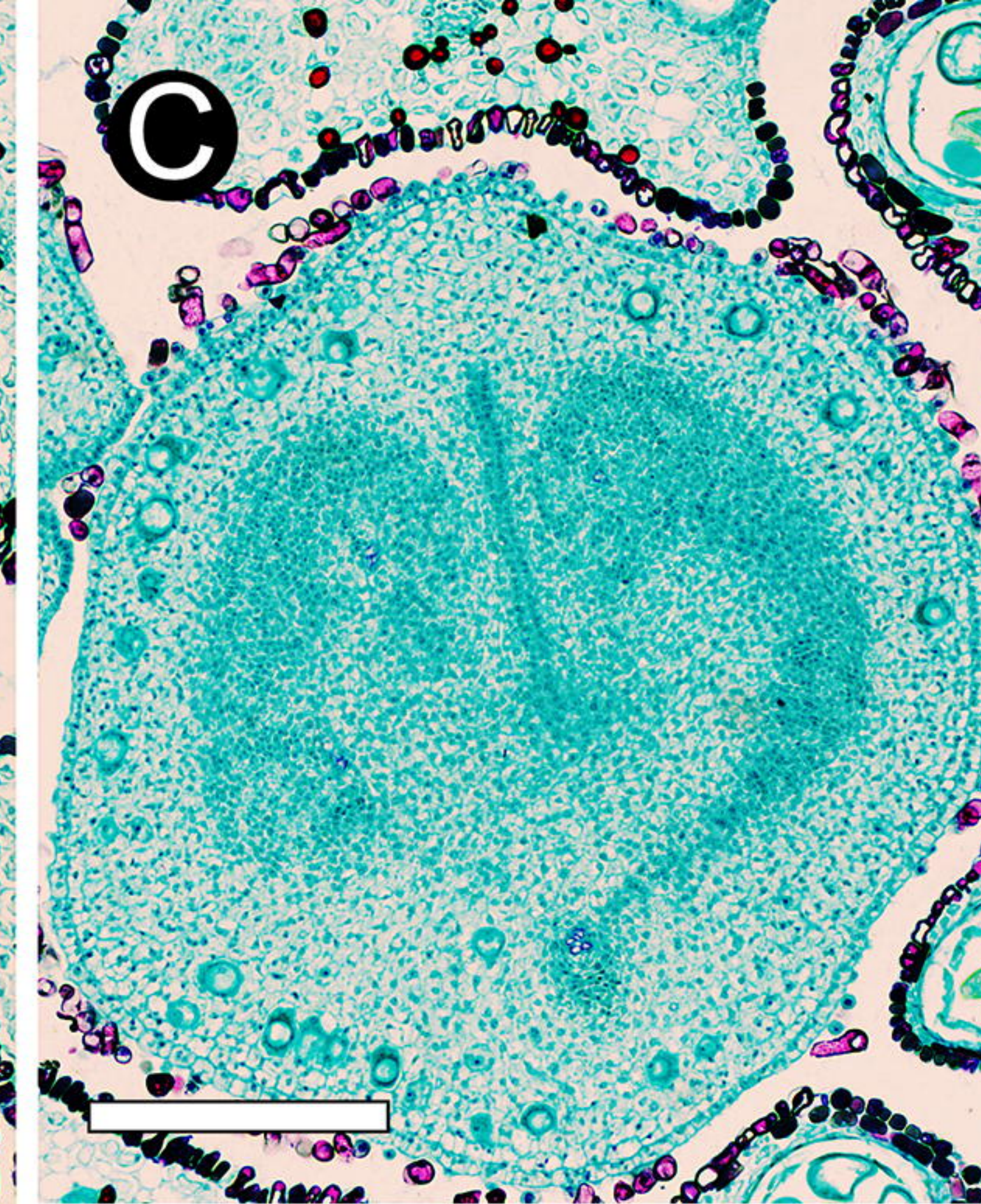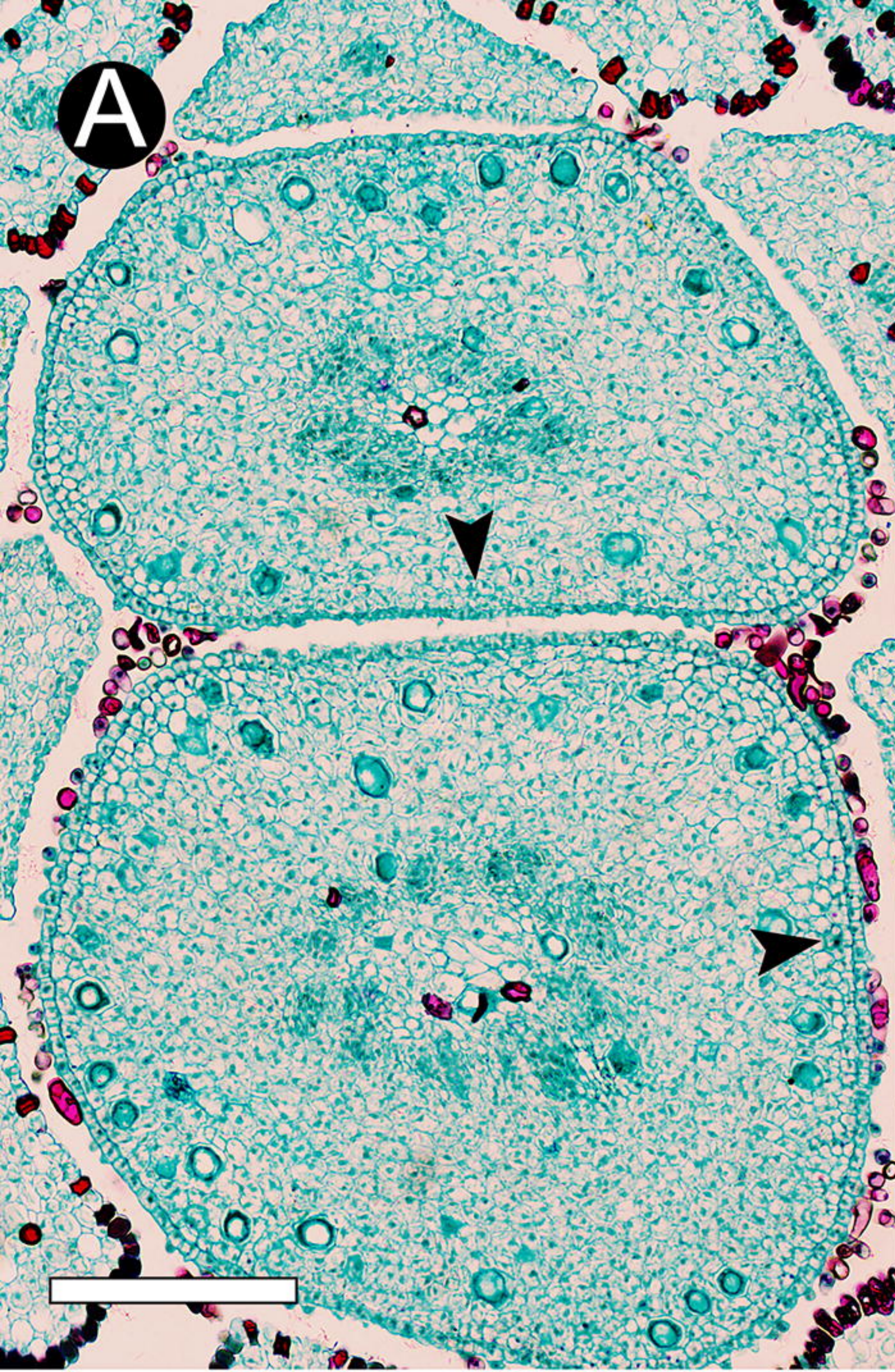480   doi: 10.11646/phytotaxa.416.3.1
481

482   **FIGURE 1.** SEM of *Anaxagorea* carpel. **(A–G)** *A. luzonensis.* **(A)** Carpel primordia.
483   **(B–C)** Appearance of the basal stipe. **(D)** Incomplete stipe closure. **(E–F)** Carpel
484   thickening and stigma formation. **(G)** Carpel stipes at anthesis (box). **(H–K)** *A.*
485   *javanica* shows similar carpel developmental features correlating with changes
486   depicted in **(A, C, D, F)**. Carpel stipes are marked by arrows. Abbreviations: c, carpel;
487   s, stamen; p, perianth. Scale bars: **(A–F, H–K)** = 100 μm; **(G)** = 1 mm.
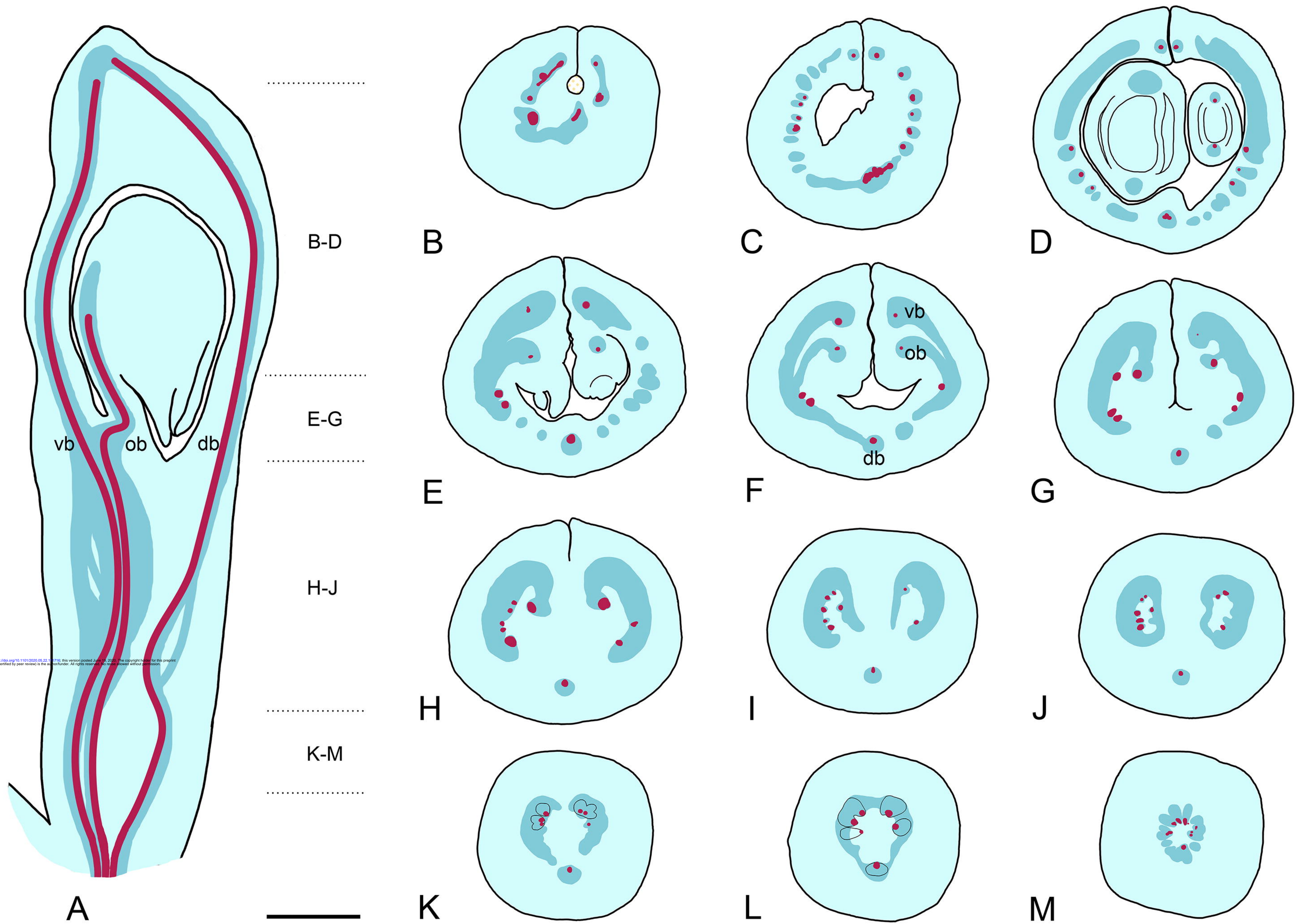488
489   **FIGURE 2.** Paraffin sections of carpels stained with Safranin O and Fast Green.
490   **(A–D)** Anthetic carpels of *A. luzonensis*, from base up. **(A)** The ring-arranged
491   collateral bundles (RACBs) at the base of carpel, ventral sides are marked by arrows.
492   **(B)** Bundle complexes with xylem surrounded by phloem in each carpel (ring marked).
493   **(C)** A short ascidiate zone. **(D)** Ovule bundles separate from the CBCs. **(E–H)** Mature
494   carpel of *A. luzonensis*. **(E)** Two sets of RACBs at the level of **(B)**. **(F)** Ventral
495   bundles (vb) and ovule bundles (ob). **(G)** Detailed view of a vb from **(F)**. **(H)** Detailed
496   view of an ob from **(F)**. **(I–M)** Mature carpel of *A. javanica*, from base up. **(I)** RACBs
497   at the base of carpel, a distinct collateral bundle marked by ring. **(J–K)** Bundles
498   rearranged into two sets of RACBs. **(L)** The CBCs with phloem joint each other. **(M)**
499   The base of ovule locule. Abbreviations: db, dorsal bundle; vb, ventral bundle; ob,
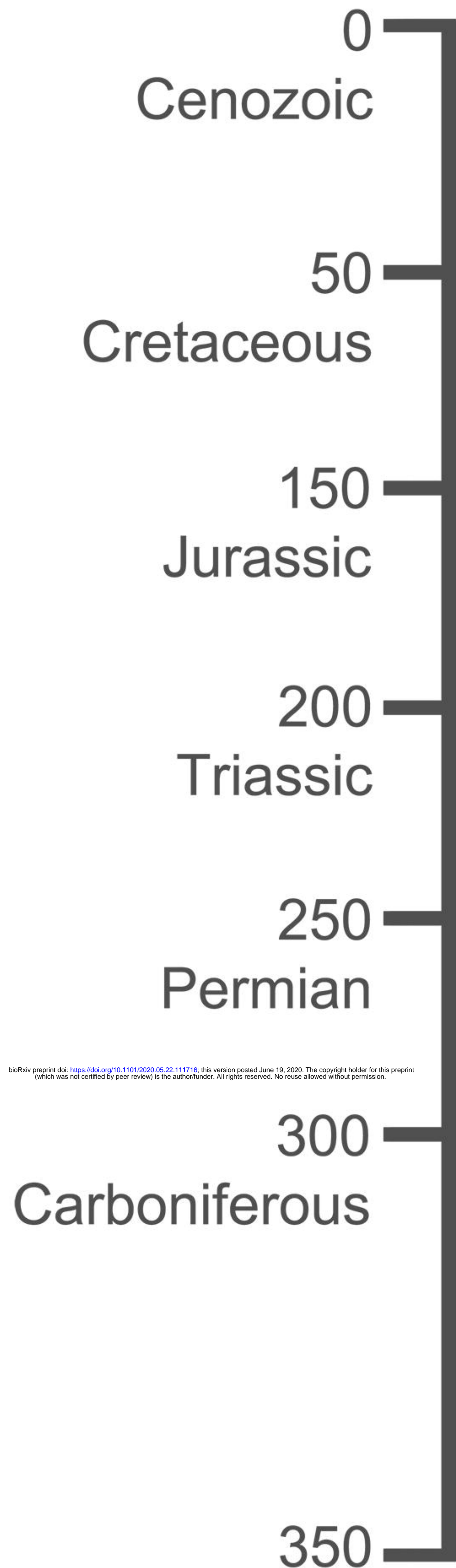500   ovule bundle. Scale bars = 200 μm.
501
502   **FIGURE 3.** Drawings of sequential sections of a mature *Anaxagorea* carpel.
503   Vasculature colored with phloem dark green and xylem red; distinct vascular bundles
504   within the vascular complex are outlined. **(A)** Schematic longitudinal section with
505   ventral side at the left. Vertical zones labeled with letters correspond to the transverse
506   sections of panels to the right. **(B–M)** Transverse sections (ventral side up) here are
507   described from the base up to the carpel in the following. **(M)** The ring-arranged
508   collateral bundles (RACBs) at the base of carpel. **(J–L)** Vasculature changes from a
509   set of RACBs at the base of the carpel to two sets of RACBs. **(H–I)** Vasculature
510   changes from each set of RACBs to the "C"-shaped bundle complexes (CBCs). **(E–G)**
511   Ovule bundles are separated from the CBCs, while others into the carpel wall. **(B–D)**
512   Meshy bundles in the carpel wall. Abbreviations: db, dorsal bundle; vb, ventral bundle;
513   ob, ovule bundle. Scale bars = 500 μm.
514
515   **FIGURE 4.** Homology comparison between *Anaxagorea* carpel vasculature and
516   female reproductive shoot of seed plants. The color implies homologies. Cordaitales
517   is a Carboniferous to Permian gymnosperm group. On the female reproductive shoot
518   of Cordaitales, the ovules (yellow) are interspersed with sterile scales (light green),
519   and ovular axis bearing bracteoles (yellow). The bracteoles on the ovular axis may
520   aggregate to the ovule and the ovular axis become shorter. Arrangement of the ovules
521   on the shoot apex (dark green) and the of lengths ovular axis are relatively flexible.
522   *Ephedra* as a genus represents gnetophytes, which shows its female reproductive
523   shoot with subtending bracts (light green), have a pair of ovules (yellow) at their
524   apical end with the abortive apex of the shoot axis (dark green) lying in between. The
525   axis bearing a pair of ovules and their envelope is called gynocladal axis.
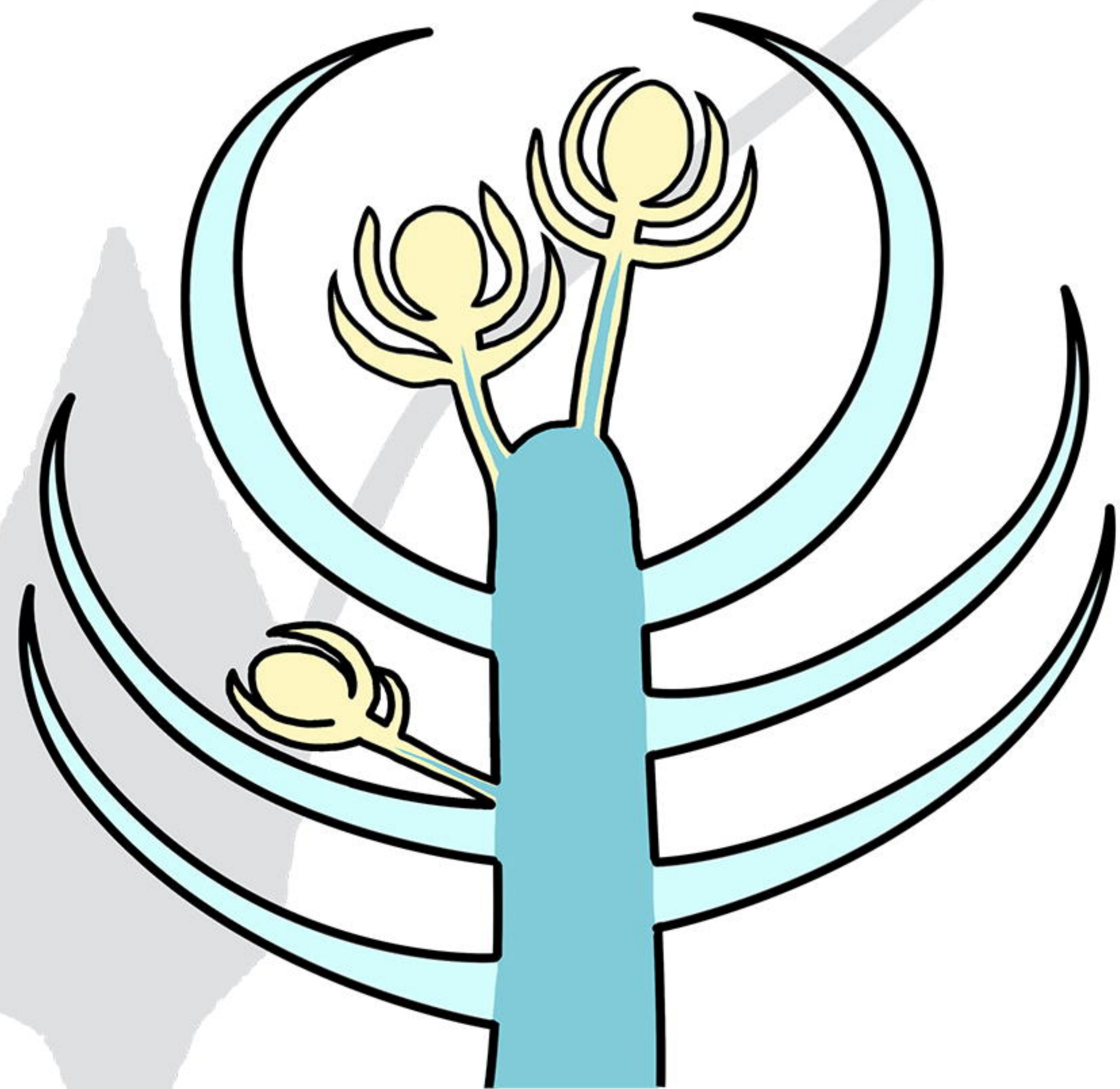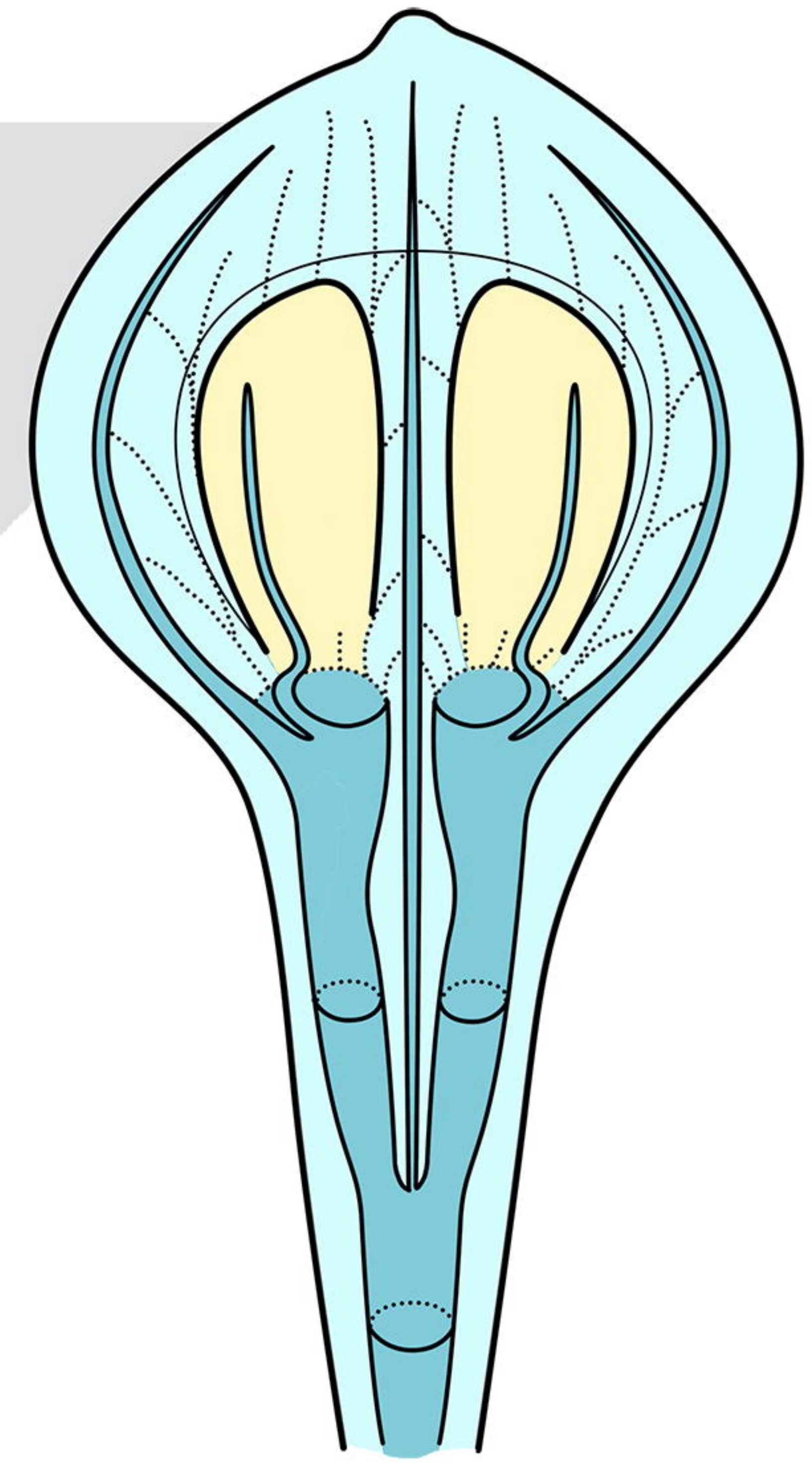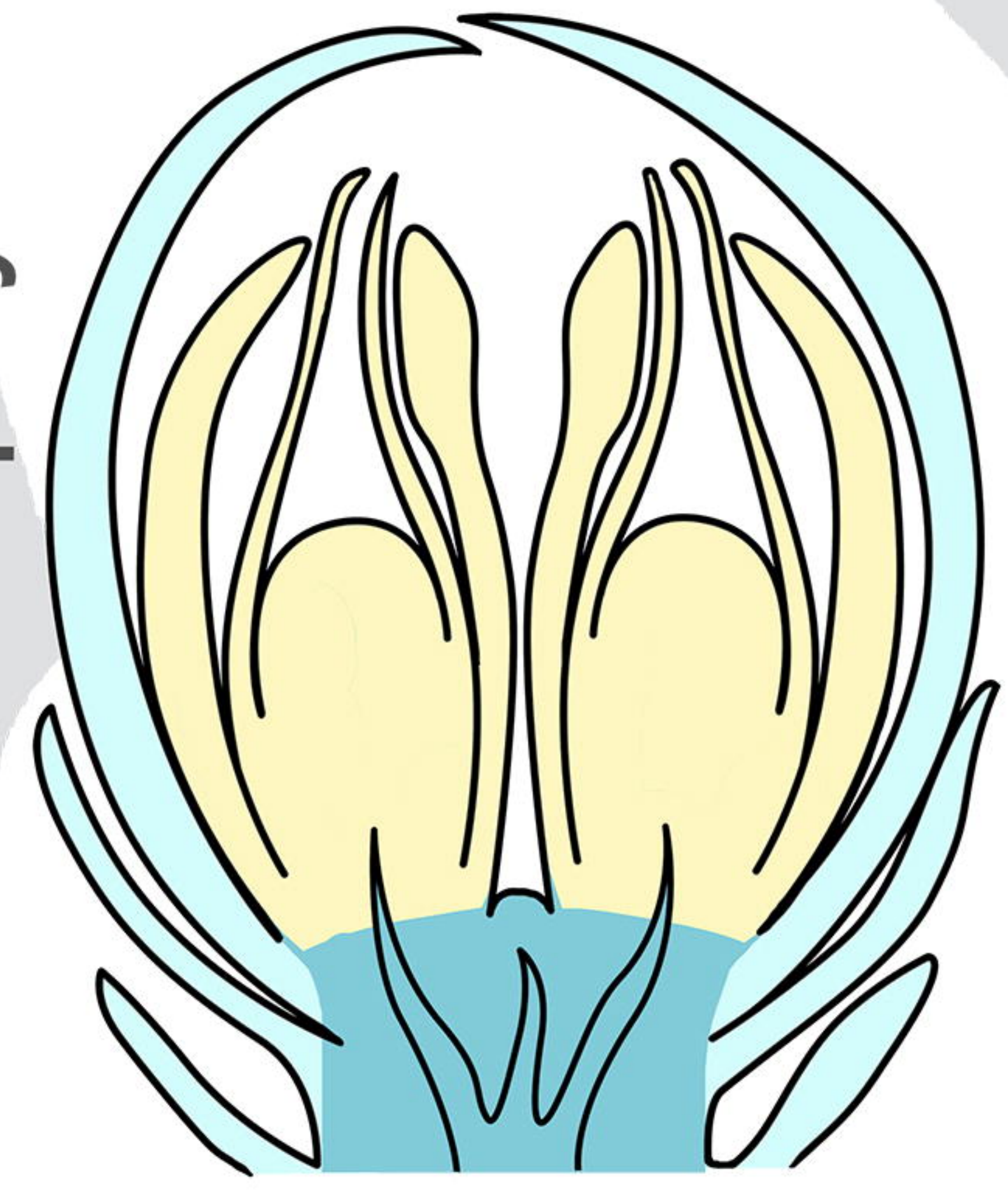
14

A

B-D

E-G

H-J

K-M

vb   ob   db

B   C   D

E   F   G

vb
ob

db

H   I   J

K   L   M

Cenozoic

0

50

Cretaceous

150

Jurassic

200

Triassic

250

Permian

300

Carboniferous

350
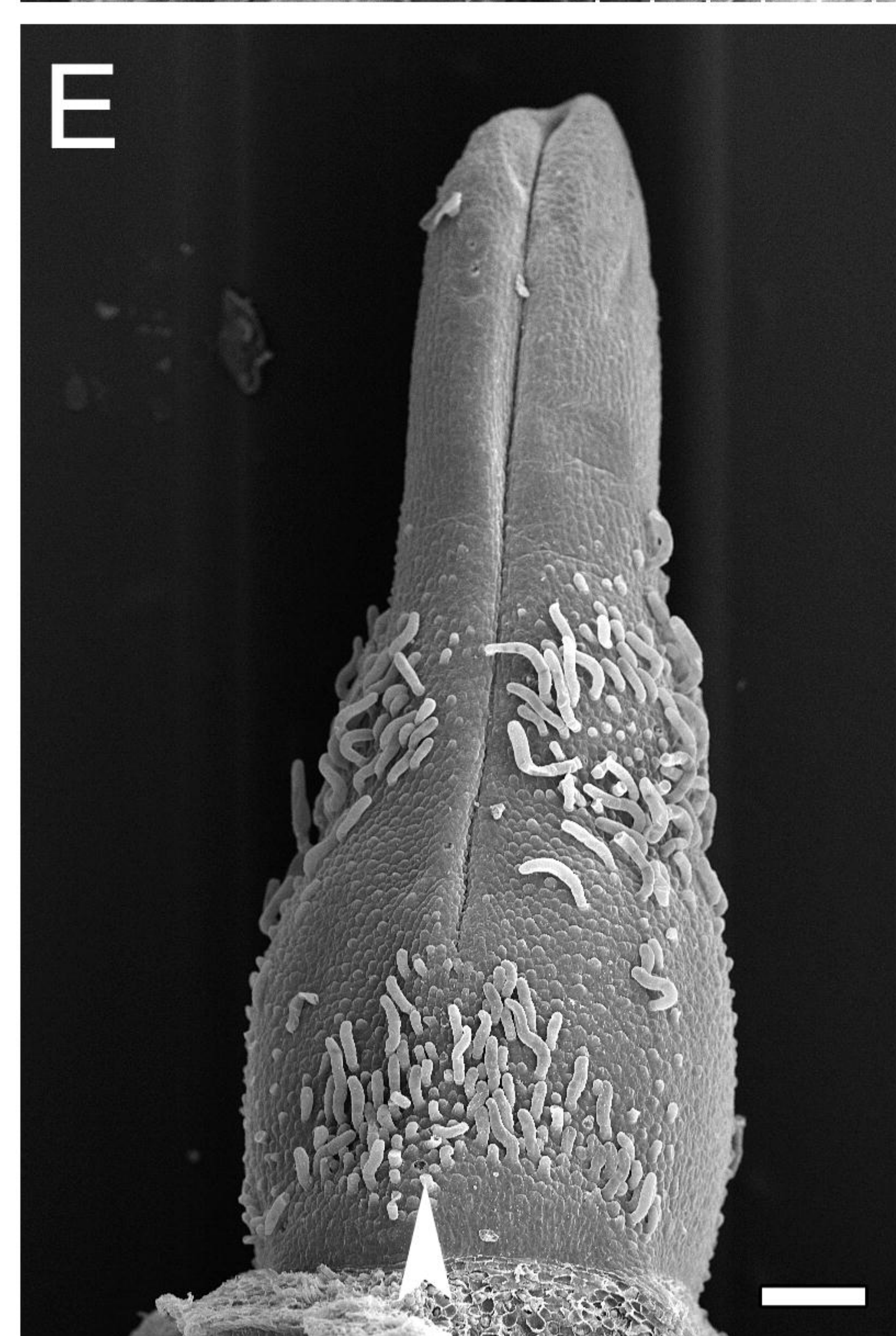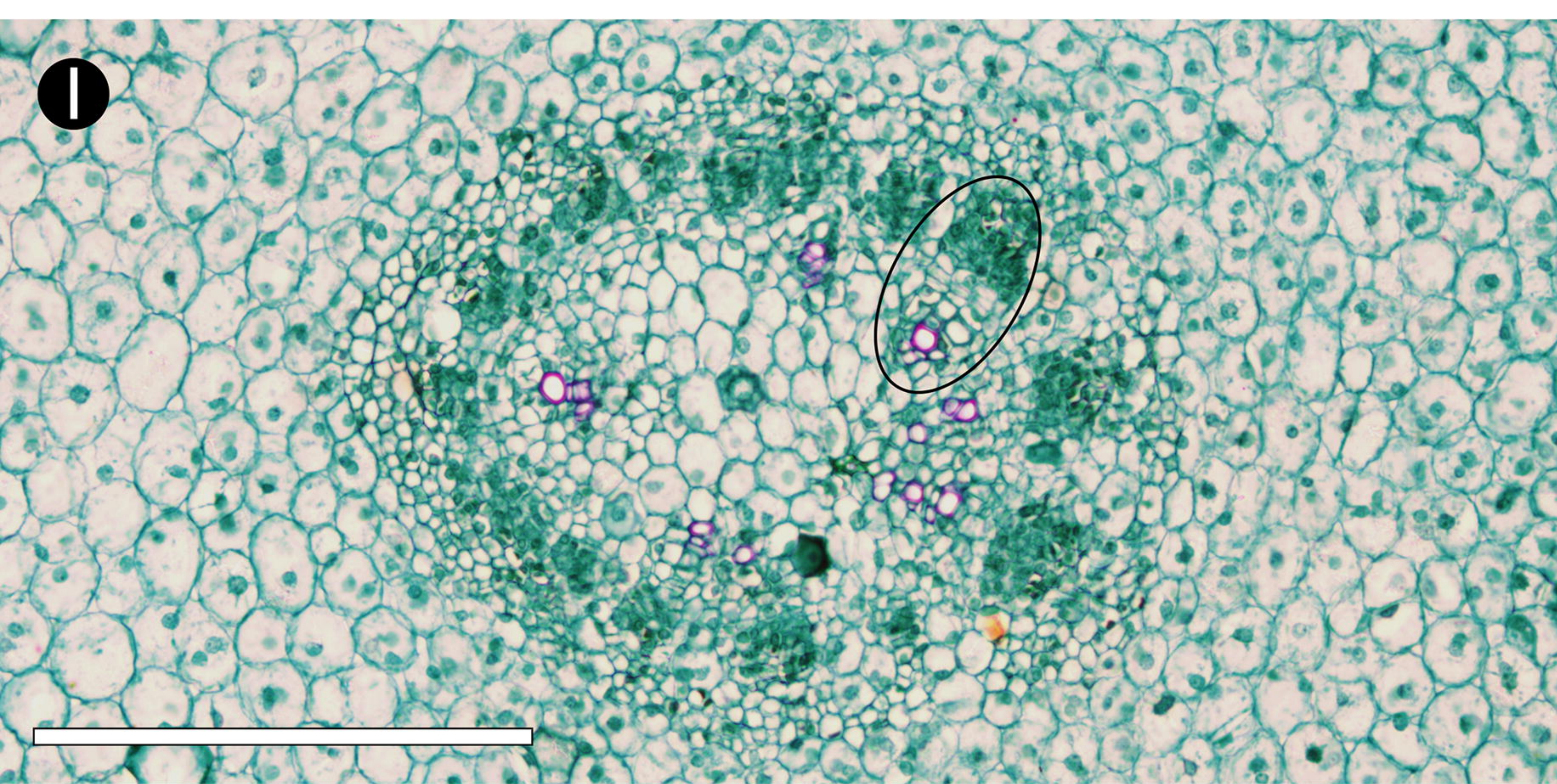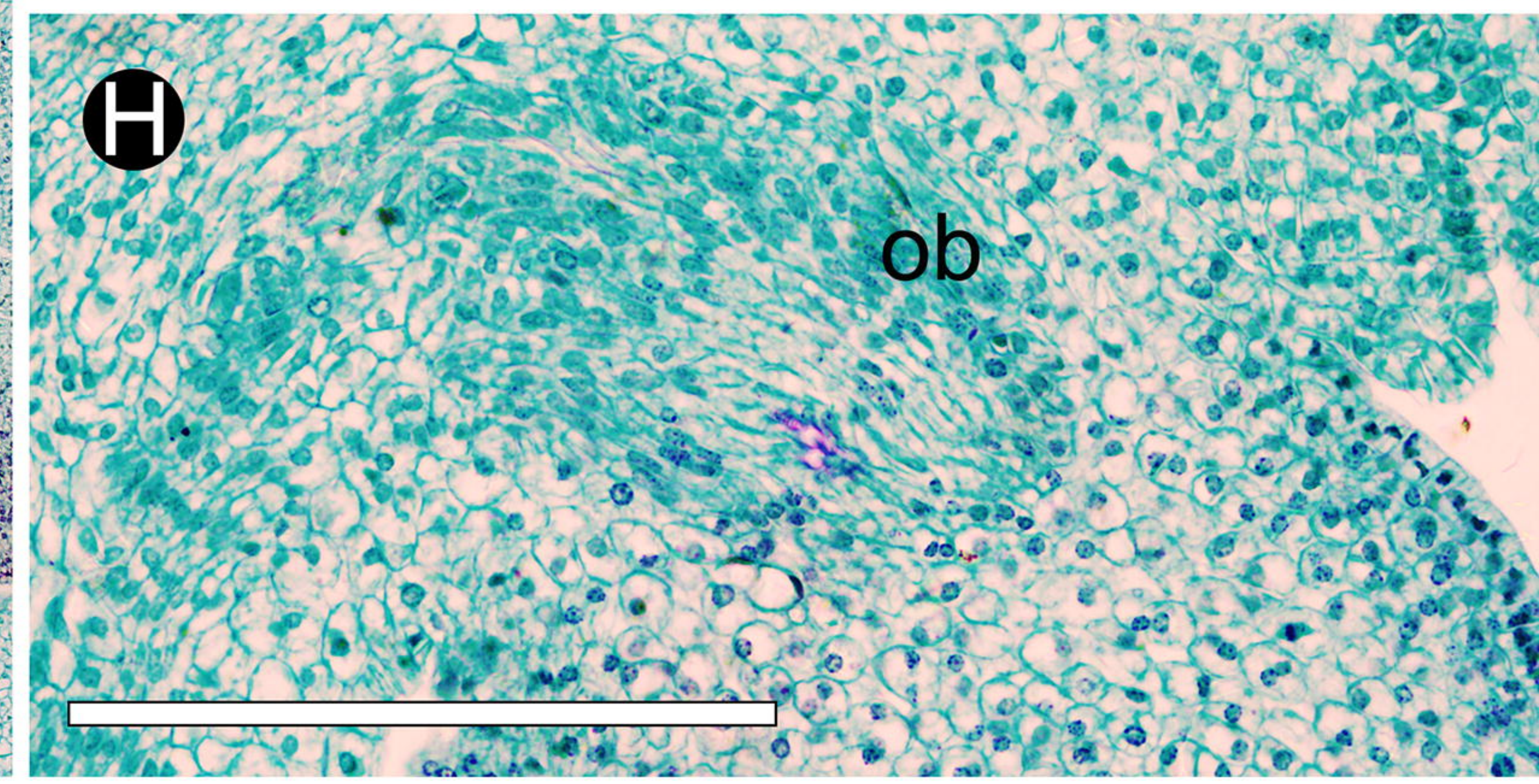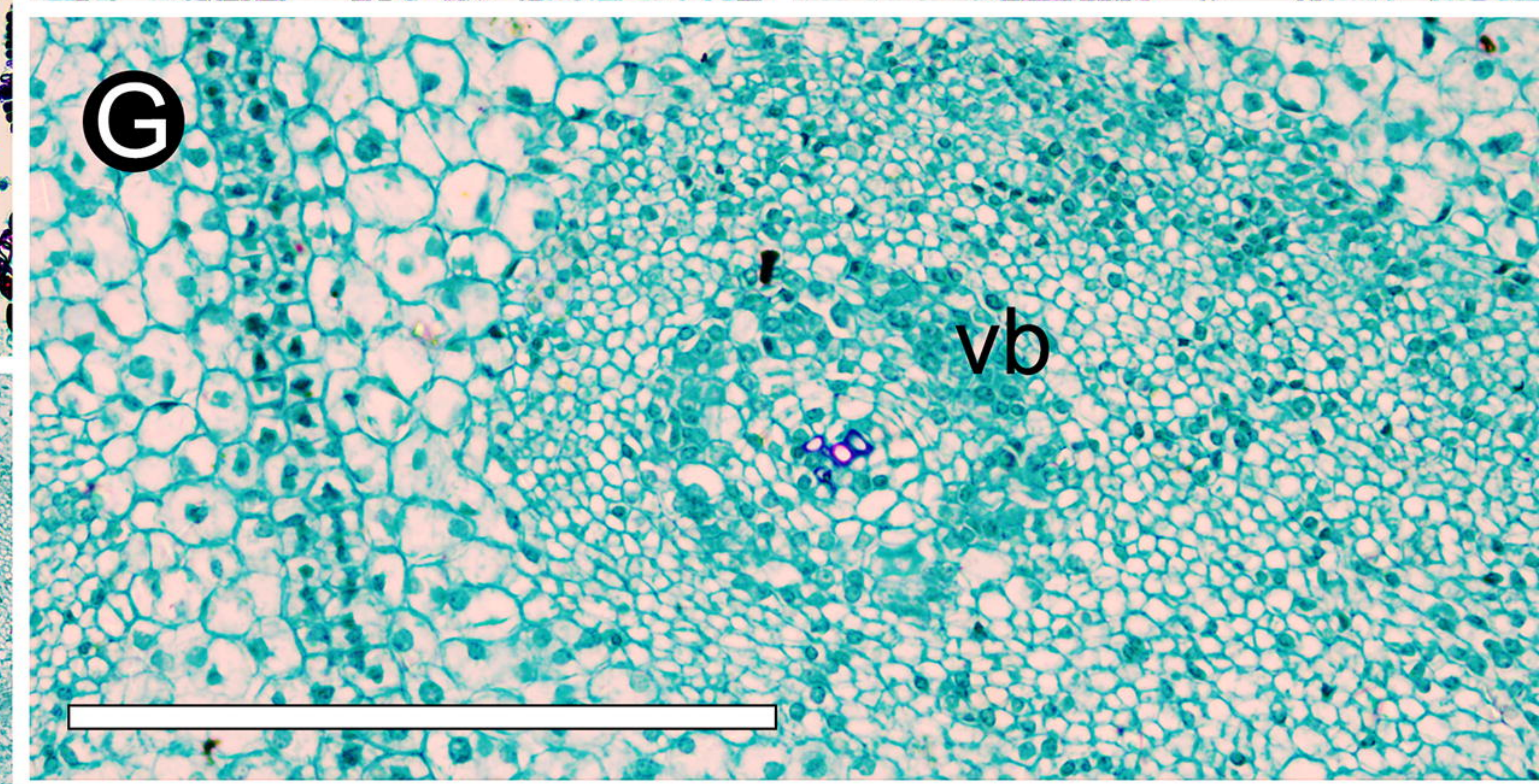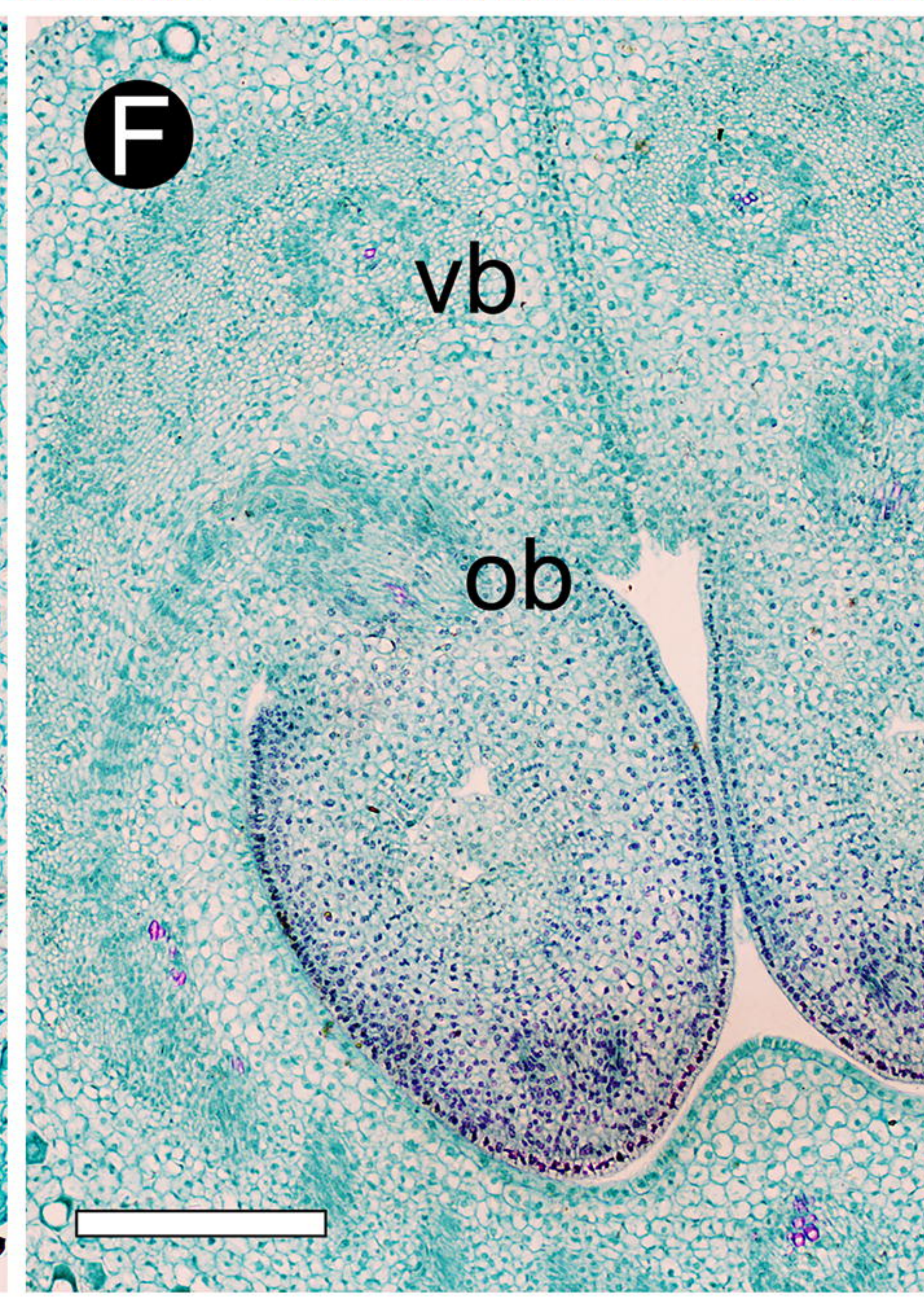
Conifers

Gnetophytes

Angiosperms

Ephedra

Anaxagorea

Cordaitales

ovule (with bracts)

shoot homologs

leaf homologs