1   **Organogenesis and Vasculature of *Anaxagorea* Revealing the Axial Homologs in**

2   **the Carpel and its Implications for the Origin of Angiosperms**

3

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10

11   Running Title:

12   Axial Homologs in Carpel and its Implications for the Origin of Angiosperms

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1 **Abstract**

2 **Background and Aims:** The carpel is the definitive structure of angiosperms, the

3 origin of carpel is of great significance to the phylogenetic origin of angiosperms.

4 Traditional view was that angiosperm carpels were derived from structures similar to

5 macrosporophylls of pteridosperms or cycads, which bear ovules on the surfaces of

6 foliar organs. In contrast, other views indicate that carpels are originated from the

7 foliar appendage enclosing the ovular axis. One of the key differences between these

8 two conflicting ideas lies in whether the "axial homologs" are involved in the

9 evolution of carpel. The aim of this paper is to investigate whether the "axial

10 homologs" remain in the carpel of extant angiosperms.

11 **Methods:** *Anaxagorea* was used for organogenesis and comparative anatomical study

12 due to its outstanding and unusually long carpel stipe. The continuous change of

13 vascular bundles at carpel stipe in different developmental stages was described in

14 detail.

15 **Key Results:** Organogenesis study shows that the carpel stipe of *Anaxagorea* occurs

16 at the early stage of carpel development. Vascular bundles at the base of *Anaxagorea*

17 carpel are a set of discrete ring-arranged collateral bundles (RACBs), which branch

18 out at the upper portion into two sets of RACBs below each ovule.

19 **Conclusions:** The ring-arranged collateral bundles indicates a clear morphological

20 evidence for the existence of the "axial homologs" in the carpel and thus support the

21 idea that carpels originated from the integration of the ovular axis and foliar parts.

22 This finding may promote reconsiderations regarding the origin of the carpel and

1   facilitate a greater understanding of the phylogenetic relationship between

2   angiosperms, gnetophytes and Cordaitales.

3

4   **Key words:** *Anaxagorea*; axial homologs; carpel; origin of angiosperms; vascular

5   anatomy.

1 **INTRODUCTION**

2 Angiosperms—the flowering plants— make up much of the visible world of modern

3 plants. Both the beautiful flowers and the delicious fruits are closely related to human

4 life. The name "angiosperm" is derived from the Greek words angeion, meaning

5 "container," and sperma, meaning "seed." Therefore, the carpel, a "seeds container", is

6 the definitive structure of angiosperms. The carpel is an angiosperm-specific female

7 reproductive feature in flowers and is the basic unit of the gynoecium, which

8 protectively surrounds the ovules by closure and sealing along their rims or flanks

9 (Dunal, 1817; Robinson-Beers, 1992; Endress, 2015). The evolution of the carpel set

10 angiosperms apart from other seed plants, which developed ovules that are exposed to

11 the air. Since the time of Darwin, elucidating the origin of angiosperms and their

12 evolutionary success represents a primary goal in plant science. Scientists have

13 attempted to search for possible ancestors of angiosperms through phylogenetic

14 analysis based on fossil, morphological, and molecular data. In these efforts,

15 particular emphasis has been placed on assessing the ease with which the

16 ovule-bearing structures of various seed plants could be transformed into a carpel.

17

18 Mainstream theories of flower origins provide differing explanations that could be

19 divided into two competing groups based on the assumed organogenetic homologies

20 of the carpel. The "phyllosporous origin" theory suggests that the carpel is a foliar

21 homolog called "megasporophyll" and the ovule either originated directly from the

22 "megasporophyll" or transferred to the "megasporophyll" ectopically (organs borne in

1   an atypical location). Under this scenario, the flower arose from a shoot bearing a

2   series of foliar homologs (e.g., Euanthial theory [Arber and Parkin, 1907; Eyde, 1975];

3   early version of "Anthophyte" theory [Melville, 1963; Stebbins, 1974; Retallack and

4   Dilcher, 1981; Crane, 1985; Doyle and Donoghue, 1986]; the Mostly Male theory

5   [Frohlich and Parker, 2000]). In contrast, the "stachyosporous origin" theory suggests

6   that the carpel is integrated axial-foliar originated (originated from the integration of

7   the ovular axis and foliar appendage). Various hypotheses supporting the

8   "stachyosporous origin" theory assumed different ancestors for angiosperms. Some

9   hypotheses suggest that the placenta in the carpel is homologous with a female

10   short-shoot of gymnosperms with distally placed ovules, each with scales forming the

11   outer integument. It follows then that the angiosperm carpel is the result of a trend

12   towards the continual reduction in the number of scales and ovules and increased

13   protection of the ovules by the surrounding organs (e.g., Pseudanthial theory

14   [Wettstein, 1907, 1935; Eames, 1952]; Neo-Pseudanthial theory [Nixon *et al.*, 1994;

15   Hickey and Taylor, 1996]; the Unifying Theory [Wang, 2010, 2018]). Another

16   hypothesis holds that the cupule wall of seed ferns provides a homolog for the outer

17   integument of angiosperm ovule and that expansion and folding of the cupule-bearing

18   axis became the progenitor of the carpel (e.g., Caytonialean hypothesis [Doyle, 1978,

19   2006, 2008]). Despite assumptions for different ancestors of angiosperms,

20   "stachyosporous origin" theory suggests that flowers derived from a compound shoot,

21   that is, from an axis that bears leaf homologs, which in turn produced axillary shoots

22   that bear ovules or microsporangia and the additional leaf homologs. However, it is

1   still difficult to reach an agreement on the origin of flowers regarding these views

2   because of (1) there is a lack of intermediate stages between the extant angiosperm

3   carpel and comparable reproductive structures in fossils, (2) the phylogenetic

4   relationships are uncertain among angiosperms and other seed plant lineages, and

5   most importantly (3) no axial ovules have been found in extant angiosperms from

6   what we know at present.

7

8   According to the above-mentioned hypotheses, the current debate focuses on whether

9   or not the "axial homologs" existence in the carpel is of great significance to the

10   origin of the carpel. Following the logic that there is no doubt the carpel wall is

11   derived from "foliar homologs", as long as the "axial homologs" in the carpel are

12   determined, the carpel should be axial-foliar originated as the "stachyosporous origin"

13   theory suggested. Recently, some vascular anatomy studies (e.g., *Actinidia* [Guo et al.,

14   2013]; *Magnolia* [Liu et al., 2014]; *Dianthus* [Guo et al., 2017]; *Michelia* [Zhang et

15   al., 2017]) proposed that the vascular system of placenta is independent to that of the

16   ovary wall in the angiosperm carpel. Vascular bundles appear amphicribral (an

17   amphicribral bundle has its xylem surrounded by the phloem) in the placenta, whereas

18   they are collateral (a collateral bundle has adaxial xylem and abaxial phloem) in the

19   ovary wall. Collateral bundles are typical arrangements of vascular bundles in

20   angiosperms (Evert and Eichhorn, 2011). In different organs, the distribution of the

21   collateral bundles is dissimilar. On the cross section of the stem in most angiosperms

22   (other than monocots), discrete collateral bundles form a single ring (hereinafter

1   abbreviated as ring-arranged collateral bundles, RACBs) around the pith. In leaves

2   (other than monocots), the largest collateral bundle often extends along the long axis

3   of the leaf as a midvein, from which smaller veins diverge laterally (Metcalfe and

4   Chalk, 1979; Evert, 2006; Beck, 2010; McKown and Dengler, 2010). Comparatively,

5   amphicribral bundles are frequently seen in small branches of early land plants, in

6   monocots, or in young branches of dicots as simplification and combination of the

7   RACBs (Fahn, 1990). The fact that vascular bundles of the placenta are independent

8   from the ovary wall, and the placenta bundles are amphicribral might provide

9   possibility for the "stachyosporous origin" of the carpel. However, the combination of

10   these two lines of evidence are still insufficient because of the flexibility of vascular

11   bundles, which brings some controversy (Endress, 2019). We believe it is possible to

12   find a more appropriate material from which extant angiosperms show clearer

13   evidence for the existence of "axial homologs" via anatomical study.

14

15   In this study, the carpel of extant angiosperm *Anaxagorea* (Annonaceae) was selected

16   for organogenesis and vascular anatomic examination, aimed at finding dependable

17   "axial homologs" as evidence for the integrated axial-foliar origin of the carpel.

18   Annonaceae are one of the largest families of Magnoliales, which is one of the most

19   important lineages in the early radiation of angiosperms (Sauquet et al., 2003).

20   *Anaxagorea* is phylogenetically the most basal genus in Annonaceae (Doyle and le

21   Thomas, 1996; Doyle et al., 2004), which live in dimly-lit riparian zones in forest

22   slope understory habitats. In this genus, plants have simple leaves that are alternately

1   arranged in two rows along the stems and the flowers usually have whorled

2   phyllotaxis. Gynoecium are apocarpous (free carpels) throughout the life history

3   (Deroin, 1988). Each carpel of *Anaxagorea* has a notable long stipe (Endress and

4   Armstrong, 2011), which is one of the important characteristics of the genus. Our

5   investigation is based on the considerations that (1) morphological features shared by

6   *Anaxagorea* are relatively primitive in angiosperms, (2) the presence of the notable

7   carpel stipe makes it possible to determine whether there are "axial homologs" in the

8   carpel through tissue sectioning.

9

10   **MATERIALS AND METHODS**

11   *Study species and sites*

12   Flower samples of *Anaxagorea luzonensis* and *Anaxagorea javanica* were collected in

13   July 2017 at the Diaoluo Mountain (Hainan, China) and Xishuangbannan Tropical

14   Botanical Garden (Yunnan, China), respectively. The former usually has two to four

15   carpels, whereas the latter has a multicarpellate gynoecium.

16

17   *SEM and paraffin slices*

18   Flower samples were collected at different floral stages (from early bud to young

19   fruit). Gynoeciums were isolated from the other parts of the flower and preserved in

20   formalin–acetic acid–70% alcohol (5:5:90, v/v). The fixed material was dehydrated

21   through a series of alcohol solutions ranging from 50% to 100%. To delineate the

22   structure and development of carpel, some carpels were removed from the

1   gynoeciums and passed through an iso-pentanol acetate series (SCR, Shanghai,

2   China), and then critically point-dried, sputter-coated with gold, and were observed

3   and photographed using a scanning electron microscope (Tescan VEGA-3-LMU,

4   Brno, Czech). To illustrate the vasculature of carpel, some carpels were embedded in

5   paraffin, sectioned serially into sections of 10- to 12-µm thickness, and then stained

6   with Safranin O and Fast Green. Complete transverse and longitudinal series were

7   examined and photographed under a light microscope (Olympus BX-43-U, Tokyo,

8   Japan) and bright field optics.

9

10   **RESULTS**

11   *Carpel organogenesis*

12   In *A. luzonensis* and *A. javanica*, carpels are free from each other completely (Figs.

13   1G, 2A). Each carpel consists of a plicate zone (Figs. 1G, 3B–H), a very short

14   ascidiate zone (Fig. 2C, here we definite ascidiate zone as a carpel region bearing

15   ovules while having no ventral slits), and a conspicuous long stipe (Figs. 1G, 3I–M).

16   Carpel primordia are approximately hemispherically initiated (Fig. 1A). Carpel stipe

17   forms early at the basal part in the early stage of carpel development (Figs. 1C, J),

18   remains elongate, takes up roughly a quarter length of the carpel at anthesis (Fig. 1G),

19   and continues to elongate during the fruit-stage. Continuous growth of the flanks on

20   the ventral side of the young carpel raises the early closure. The closure does not

21   extend to the bottom of the carpel (Fig. 1D). Subsequently, the dorsal part of each

22   carpel notably thickens and the stigma forms (Figs. 1E, F). At anthesis, carpels are

1   widest at the basal part, with an arch on abaxial side (Fig. 1G). Each carpel has two

2   lateral ovules with the placentae at the base of the ovary (Figs. 2F, M, 3A).

3

4   *Carpel vasculature*

5   Main vascular bundles of *Anaxagorea* carpel are distinct as a dorsal bundle, a pair of

6   ventral bundles, a pair of ovule bundles, and several lateral bundles (Figs. 2F, M, 3A,

7   F). Vascular system here are described from the base up to the carpel for easier

8   comprehension of vascular transformation according to the schematic (Fig. 3). At the

9   base of each carpel stipe, discrete collateral bundles form a ring (RACBs) around the

10   pith-like cell groups (Figs. 2A, I, 3M). At the slightly upper part of the carpel stipe,

11   those discrete bundles tend to join adjacent bundles in phloem (Fig. 3L), subsequently

12   separate, and finally are gathered into three groups: a dorsal bundle and two sets of

13   lateral bundle complexes (LBCs, Fig. 3K). Each set of LBCs is assembled into an

14   amphicribral bundle in the young carpel (Fig. 2B). While in the mature carpel, each

15   set of LBCs is assembled as a set of RACBs, and thus there are two sets of RACBs in

16   one carpel (Figs. 2E, K, 3J). Below each placenta, each set of RACBs (or

17   amphicribral bundle) is transformed to a set of "C"-shaped bundle complexes (CBCs),

18   from which several collateral bundles separate from each set of CBCs, and merge

19   with each other to form an amphicribral bundle, which is kept separate from other

20   bundles (Figs. 2C, L, 3G–I). Each set of CBCs allocates an ovule bundle into each

21   ovule and other bundles into the carpel wall, where the ventral bundles are also

22   separated (Figs. 2D, F, M, 3E–F). On the cross section of the ovary locule, both

1   ventral bundles and ovule bundles are amphicribral (Figs. 2G, H). In the carpel wall,

2   lateral bundles present as pinnate arranged collateral bundles, which are connected to

3   form meshy complexes by poorly-differentiated strands (Figs. 2M, 3D, E). The dorsal

4   bundle is also connected with lateral bundles by poorly-differentiated strands (Figs.

5   2D, 3F). Upon the ovule locule, bundles in the carpel wall join each other with xylem

6   and phloem and finally diminish (Figs. 3B, C).

7

8   **DISCUSSION**

9   *Morphological analysis*

10   Organogenesis study shows that the carpel stipe of *Anaxagorea* occurs at the early

11   stage of carpel development and continues to elongate with the development of the

12   carpel. It is different from the mericarp stipes of other Annonaceae plants, the latter of

13   which are not obvious until the fruit stage, and partly comes from the elongated

14   receptacle (e.g., *Annona*, *Cananga*, *Uvari*, *Xylopia* [Deroin, 1999]). The carpel stipe

15   in *Anaxagorea* provides a buffer space for the drastic changes in structure of the

16   vascular bundle at the base of carpel, whereas in most angiosperms with apocarpy, the

17   base of the carpel is very close to the placenta and the vascular bundle in this part

18   tends to be simplified (e.g., *Michelia* [Tucker, 1961]; *Sagittaria* [Kaul, 1967]; *Drimys*

19   [Tucker, 1975]; *Illicium* [Robertson and Tucker, 1979]; *Brasenia* [Endress, 2005]).

20   The RACBs at the basal cross sections act in accordance with a typical form of the

21   stem vascular anatomy of most angiosperms other than monocots (e.g., Caprifoliaceae;

22   Leguminosae; Ulmaceae). In the distal region, a single collateral bundle separated

1   from the basal RACBs, showed foliar homology. The remainder gathered into two

2   sets of RACBs below each placenta, which are especially obvious in the more mature

3   carpel, and showed axial homology. As we know, the vascular distribution in the

4   leaves are lateral organized in most seed plants (e.g., Pinaceae; Gnetaceae;

5   Magnoliaceae; Gramineae; Brassicaceae). Even in the branching veins in *Ginkgo*,

6   each of the veins is a single collateral bundle. The topological structure of the lateral

7   organized bundles is equivalent to a curve, and the curved surface can only form a

8   one-way tunnel (like macaroni pasta) by coiling. However, the branching RACBs

9   (one set of RACBs branch into two sets of RACBs) in the carpel of *Anaxagorea* are

10   equivalent to a three-way tunnel. The branching of RACBs in each carpel seems to be

11   reliable evidence for the existence of "axial homologs" in the carpel because the foliar

12   homologs (lateral organs) cannot form such a topology by coiling transformation.

13

14   In the carpel of *Anaxagorea*, amphicribral bundles were observed in the young carpel

15   stipe and in the funiculus of the mature ovule as simplification and combination of the

16   RACBs. The fact that the amphicribral bundles are widespread in the carpel and

17   funiculus of angiosperms (e.g., *Papaver* [Kapoor, 1973], *Psoraleae* [Lersten and Don,

18   1966], *Drimys* [Tucker, 1975], *Nicotiana* [Dave et al., 1981], *Whytockia* [Wang and

19   Pan, 1998], *Pachysandra* [Von Balthazar and Endress, 2002], *Actinidia* [Guo et al.,

20   2013], *Magnolia* [Liu et al., 2014]; *Dianthus* [Guo et al., 2017]; *Michelia* [Zhang et

21   al., 2017]) would be a reasonable result if the ovule-placenta in the carpel is

22   originated from the ovular axis. In addition, we should notice that a carpel has

1   developed as a functional entity, and there is no axis or axillary shoot in the carpel.

2   The words "axial homologs" we use are mainly to emphasize the possible

3   phylogenetic origination of the carpel.

4

5   *Homology Comparison*

6   The existence of "axial homologs" in carpels might facilitate understanding of the

7   phylogenetic origin of angiosperms by homology comparison. In outgroups, the

8   female reproductive shoots, which are similar to the branching RACBs-placenta

9   complex in the carpel of *Anaxagorea,* can be found in *Ephedra* and the extinct

10   Cordaitales-like plants (Fig. 4).

11

12   *Ephedra* is an extant gymnosperm representing gnetophytes. Neo-Pseudanthial theory

13   suggested that the angiosperm carpel arose from the foreshortening and integration of

14   the female reproductive shoot of *Ephedra* (Hickey and Taylor, 1996). Our results

15   suggest that the carpel vasculature of *Anaxagorea* has branching RACBs and is

16   comparable with the female reproductive shoot of *Ephedra*. If the branching RACBs

17   are the plesiomorphy of the common ancestor of angiosperms and *Ephedra*, then it is

18   more plausible that the carpels have stronger homology with the female reproductive

19   shoot than the cupule-bearing axis in Caytonia (Doyle, 1978, 2008) because the

20   cupule-bearing axis of Caytonia does not bear the secondary shoot. Despite the

21   phylogenetic hypothesis that *Ephedra* are the closest living relatives of angiosperms

22   in the Neo-Pseudanthial theory, this postition has currently been refuted (Hajibabaei et

1   al., 2006; Wickett et al., 2014; Wan et al., 2018). However, we should be careful not

2   to discard the homology comparison as an independent component of the theory.

3

4   Other views have provided a relatively moderate explanation that angiosperms,

5   conifers, and gnetophytes (*Ephedra*, *Gnetum*, *Welwitschia*) are derived from a

6   common ancestor in Cordaitales (Eames, 1952; Bold, 1973; Hickey and Taylor, 1996;

7   Doyle, 2008; Wang, 2010, 2018). The Unifying Theory (Wang, 2010, 2018) suggests

8   that angiosperm carpels are derived from the sterile bracts (formed carpel wall)

9   attached to the reproductive secondary shoot (formed ovule/placenta) of Cordaitales.

10   Taking Cordaitales as the common ancestor of angiosperms and other gymnosperms

11   may resolve the contradiction between the similar morphological characters and the

12   distant phylogenetic relationship between angiosperms and gnetophytes. The

13   branching RACBs of *Anaxagorea* carpels suggest that the carpel might comparable to

14   a compound shoot and provide an understanding of how the bract-bracteole-terminal

15   ovule system in Cordaitales-like gymnosperms evolved to an angiosperm carpel.

16

17   Our results provide more reliable evidence for the existence of "axial homologs" in

18   the carpel of extant angiosperms. An "axial homolog" should not appear in the

19   "megasporophyll". However, if that is true, angiosperm carpels should be integrated

20   axial-foliar originated. Nature follows the principle that selecting a simple mechanism

21   or module as building blocks for a complex system and then using that module

22   repeatedly in other systems. The presentation of "axial homologs" in carpel seems the

1   embodiment of this rule.

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1 **FIG. 1.** SEM of *Anaxagorea* carpel. (A–G) *A. luzonensis.* (A) Carpel primordia. (B–C)

2 Appearance of the basal stipe. (D) Incomplete stipe closure. (E–F) Carpel thickening

3 and stigma formation. (G) Carpel stipes at anthesis (box). (H–K) *A. javanica* shows

4 similar carpel developmental features correlating with changes depicted in (A, C, D,

5 F). Carpel stipes are marked by arrows. Abbreviations: c, carpel; s, stamen; p, perianth.

6 Scale bars: (A–F, H–K) = 100 μm; (G) = 1 mm.

7

8 **FIG. 2.** Paraffin sections of carpels stained with Safranin O and Fast Green. (A–D)

9 Anthetic carpels of *A. luzonensis*, from base up. (A) The ring-arranged collateral

10 bundles (RACBs) at the base of carpel, ventral sides are marked by arrows. (B)

11 Bundle complexes with xylem surrounded by phloem in each carpel (ring marked). (C)

12 A short ascidiate zone. (D) Ovule bundles separate from the CBCs. (E–H) Mature

13 carpel of *A. luzonensis*. (E) Two sets of RACBs at the level of (B). (F) Ventral bundles

14 (vb) and ovule bundles (ob). (G) Detailed view of a vb from (F). (H) Detailed view of

15 an ob from (F). (I–M) Mature carpel of *A. javanica*, from base up. (I) RACBs at the

16 base of carpel, a distinct collateral bundle marked by ring. (J–K) Bundles rearranged

17 into two sets of RACBs. (L) The CBCs with phloem joint each other. (M) The base of

18 ovule locule. Abbreviations: db, dorsal bundle; vb, ventral bundle; ob, ovule bundle.

19 Scale bars = 200 μm.

20

21 **FIG. 3.** Drawings of sequential sections of a mature *Anaxagorea* carpel. Vasculature

22 colored with phloem dark green and xylem red; distinct vascular bundles within the

1 vascular complex are outlined. (A) Schematic longitudinal section with ventral side at

2 the left. Vertical zones labeled with letters correspond to the transverse sections of

3 panels to the right. (B–M) Transverse sections (ventral side up) here are described

4 from the base up to the carpel in the following. (M) The ring-arranged collateral

5 bundles (RACBs) at the base of carpel. (J–L) Vasculature changes from a set of

6 RACBs at the base of the carpel to two sets of RACBs. (H–I) Vasculature changes

7 from each set of RACBs to the "C"-shaped bundle complexes (CBCs). (E–G) Ovule

8 bundles are separated from the CBCs, while others into the carpel wall. (B–D) Meshy

9 bundles in the carpel wall. Abbreviations: db, dorsal bundle; vb, ventral bundle; ob,

10 ovule bundle. Scale bars = 500 μm.

11

12 **FIG. 4.** Homology comparison between *Anaxagorea* carpel vasculature and female

13 reproductive shoot of seed plants. The color implies homologies. Cordaitales is a

14 Carboniferous to Permian gymnosperm group. On the female reproductive shoot of

15 Cordaitales, the ovules (yellow) are interspersed with sterile scales (light green), and

16 ovular axis bearing bracteoles (yellow). The bracteoles on the ovular axis may

17 aggregate to the ovule and the ovular axis become shorter. Arrangement of the ovules

18 on the shoot apex (dark green) and the of lengths ovular axis are relatively flexible.

19 *Ephedra* as a genus represents gnetophytes, which shows its female reproductive

20 shoot with subtending bracts (light green), have a pair of ovules (yellow) at their

21 apical end with the abortive apex of the shoot axis (dark green) lying in between. The

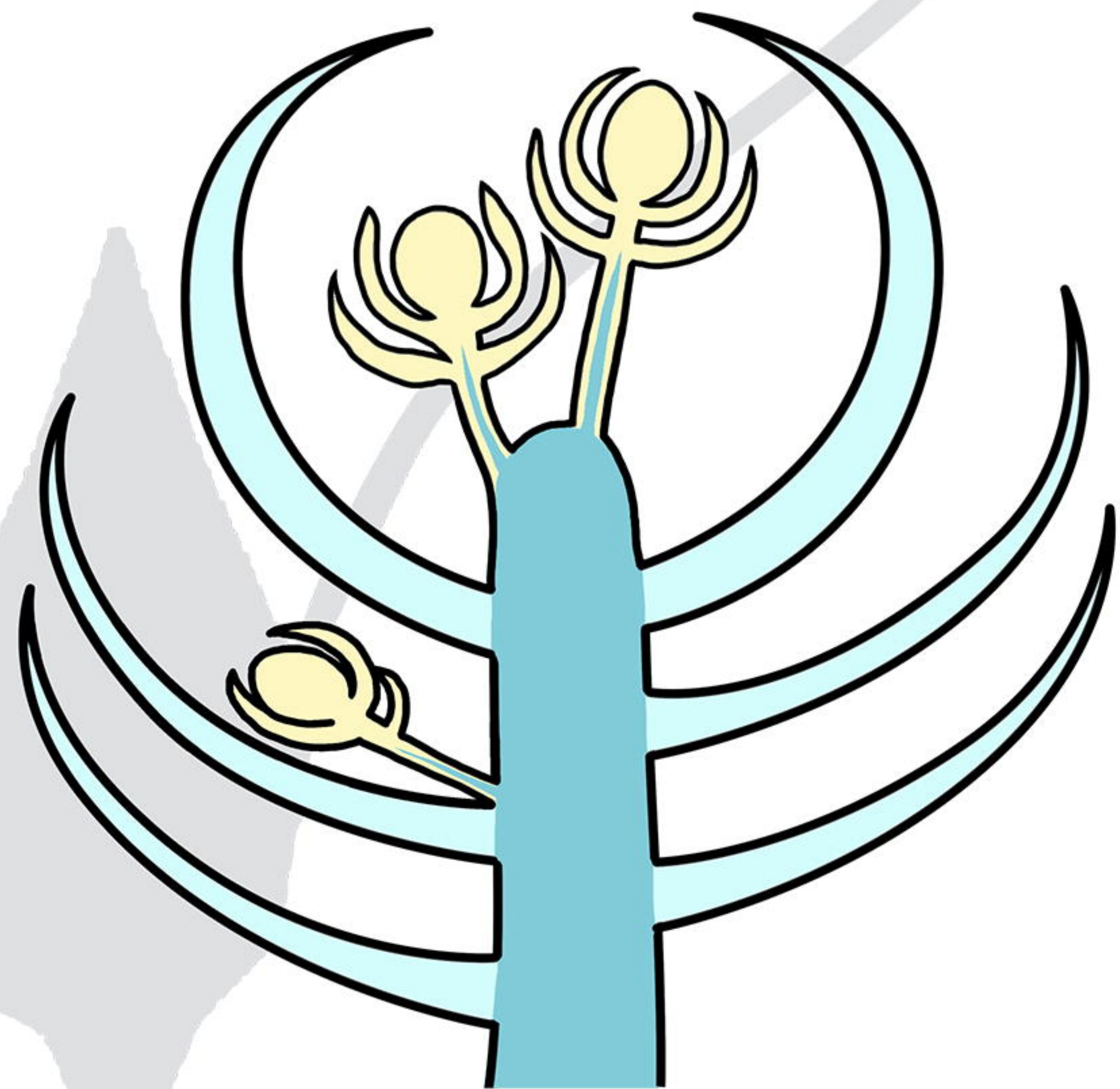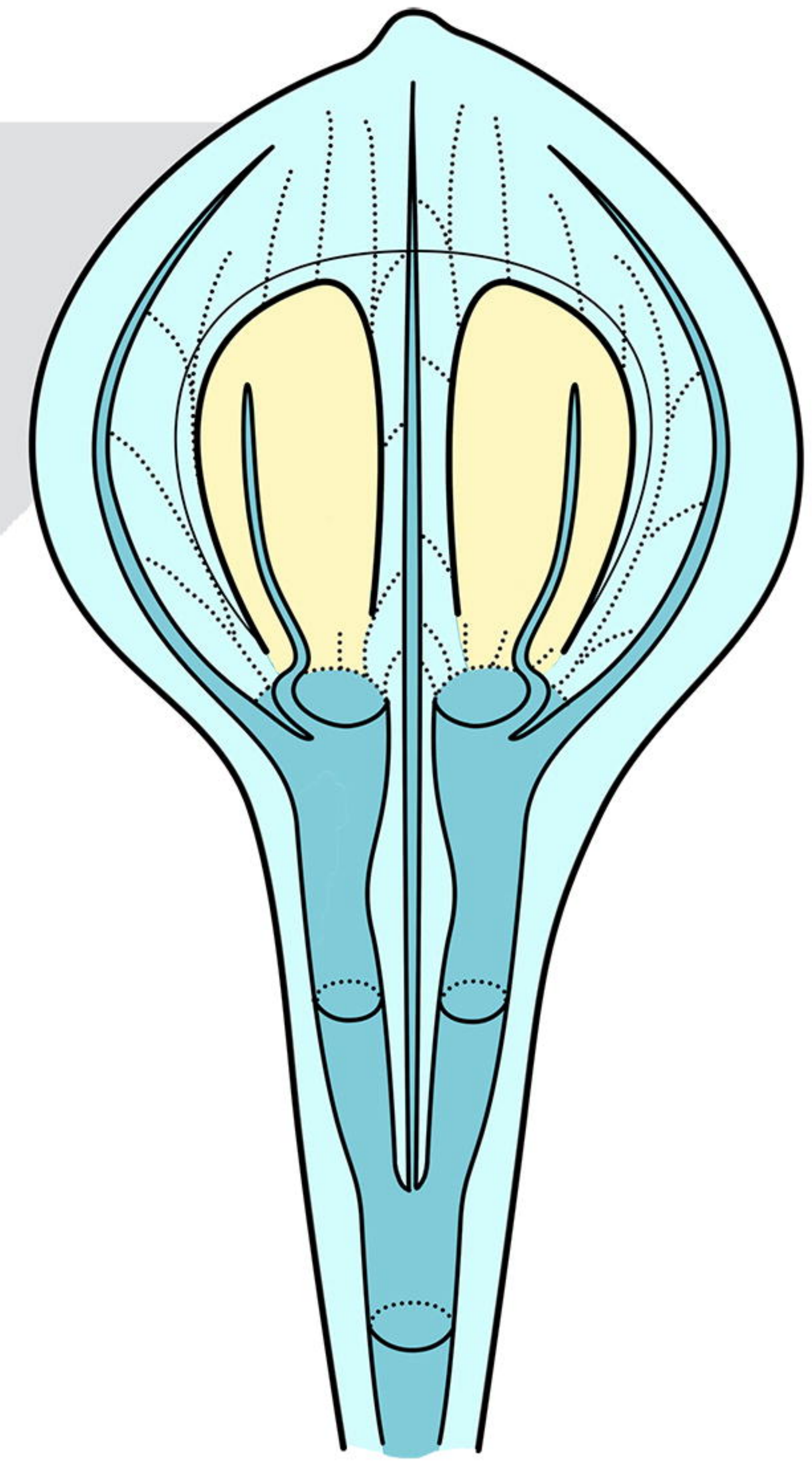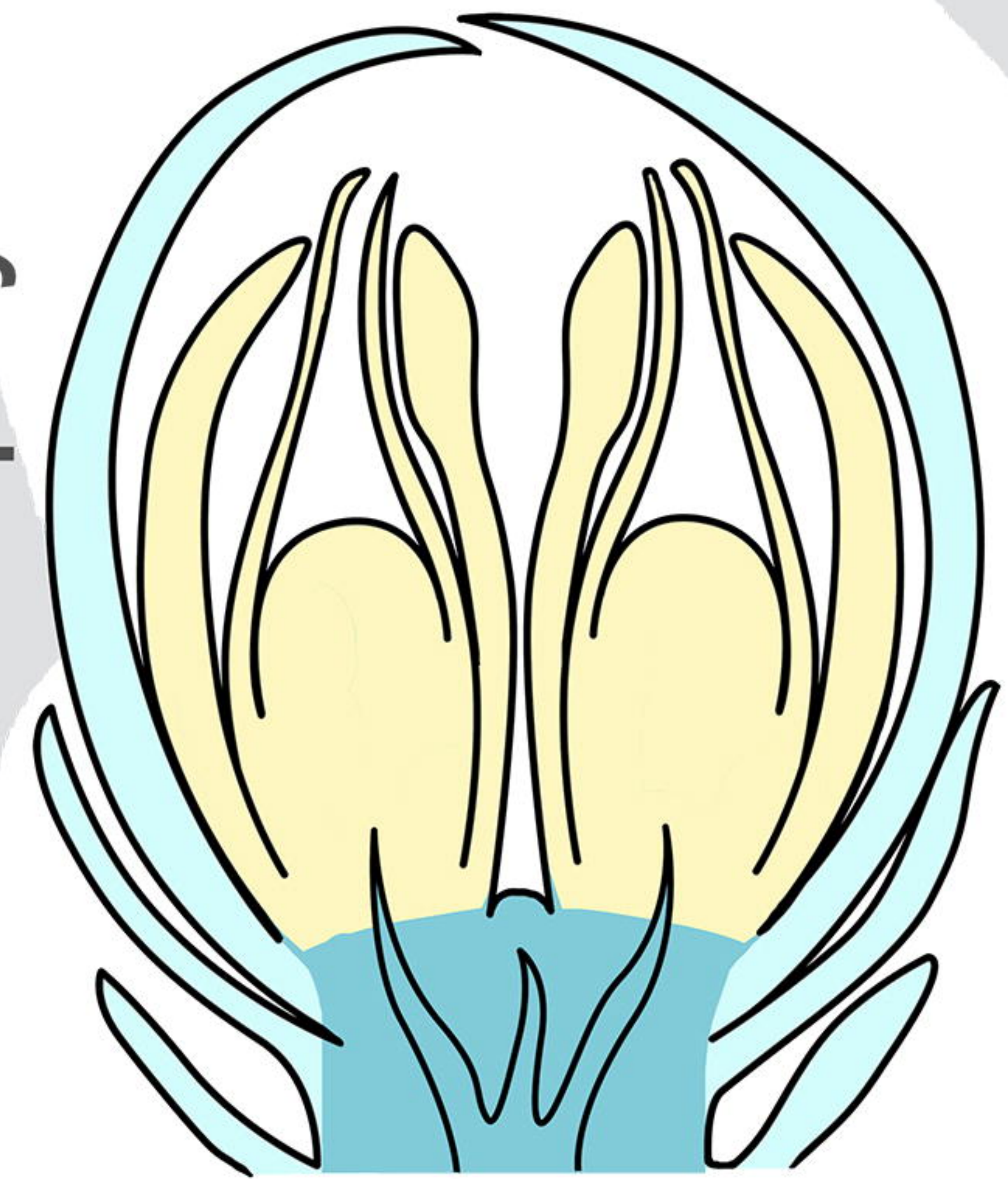22 axis bearing a pair of ovules and their envelope is called gynocladal axis.

Cenozoic

Cretaceous

Jurassic

Triassic

Permian

Carboniferous

0

50

150

200

250

300

350
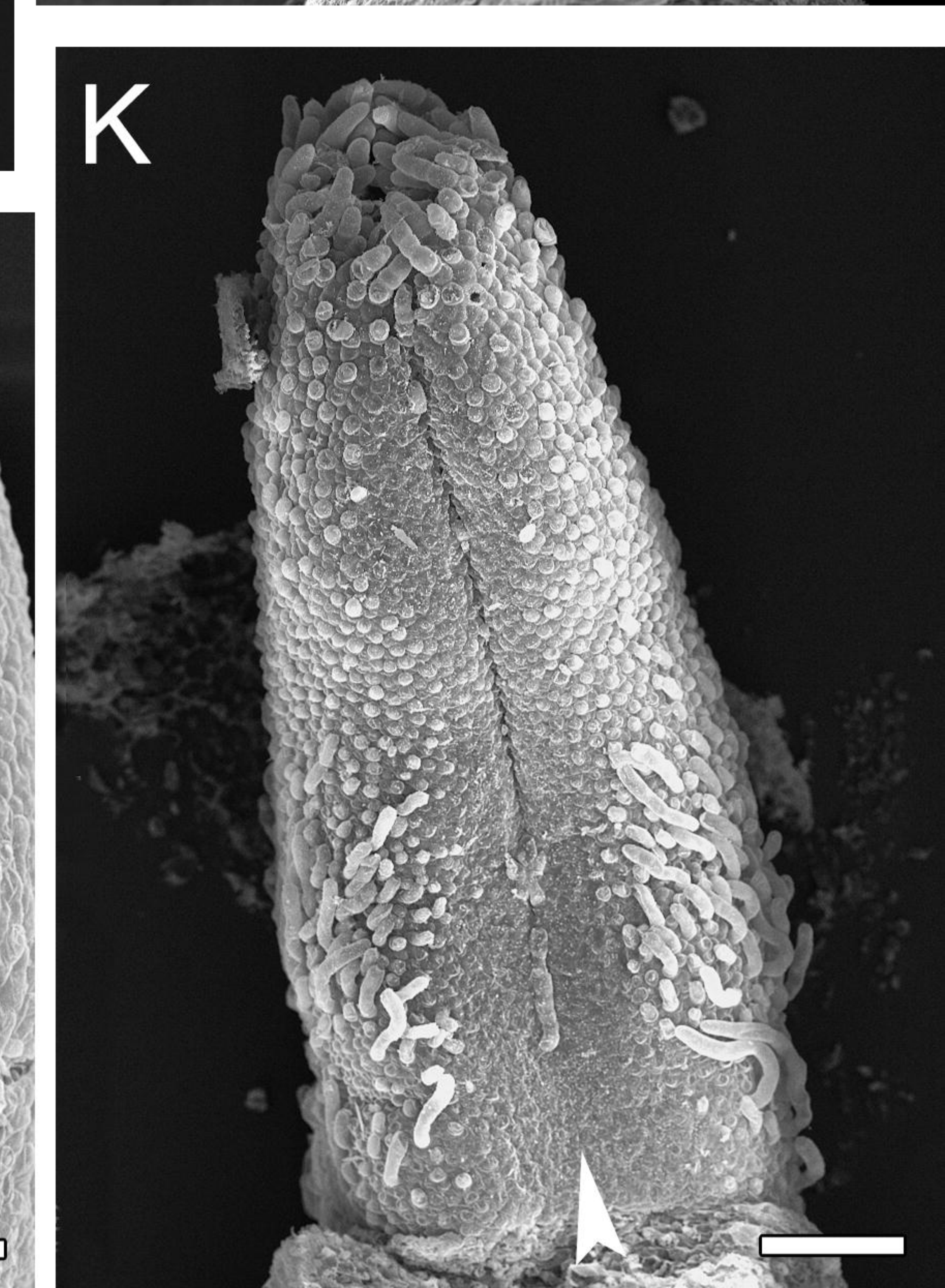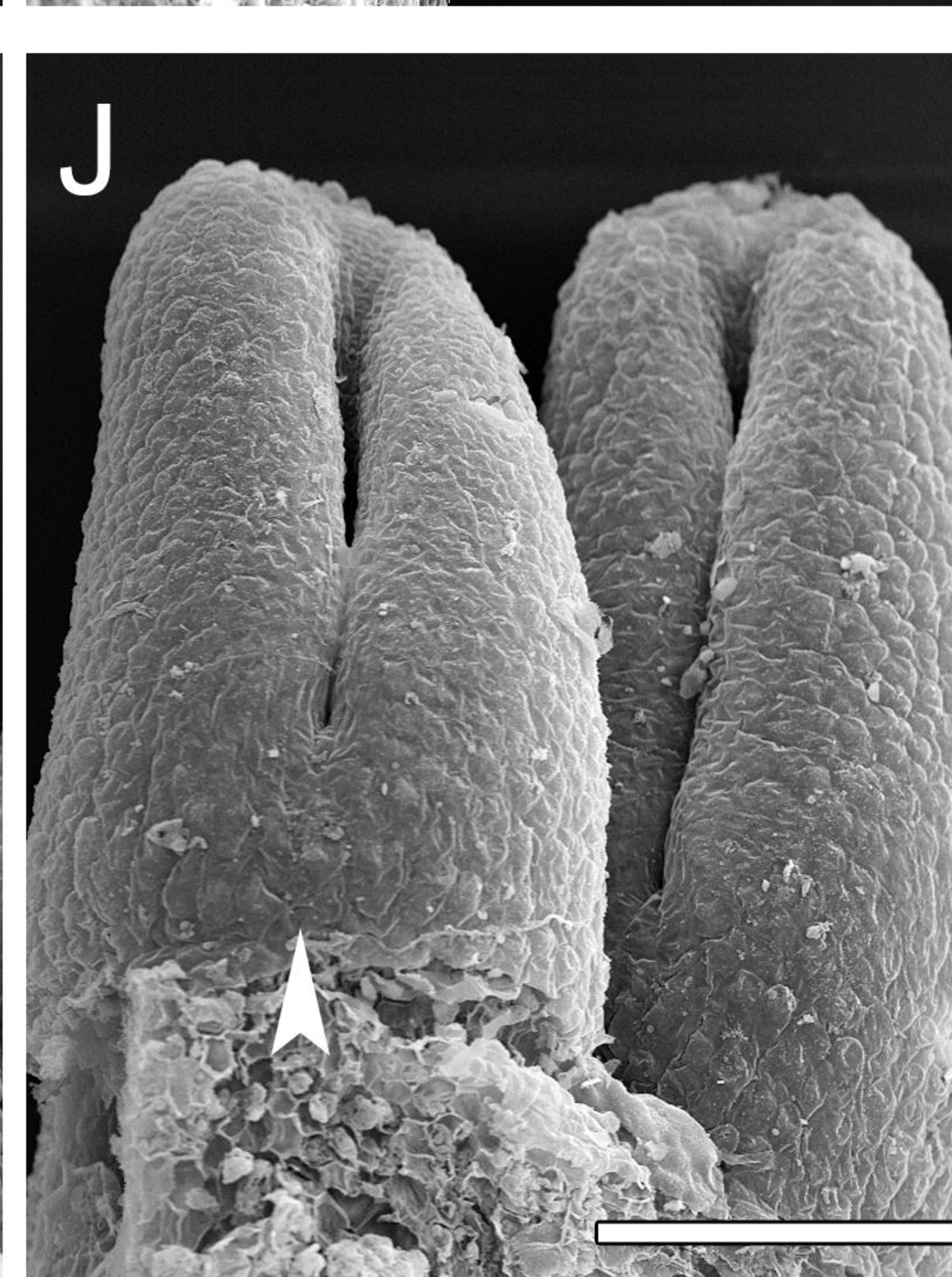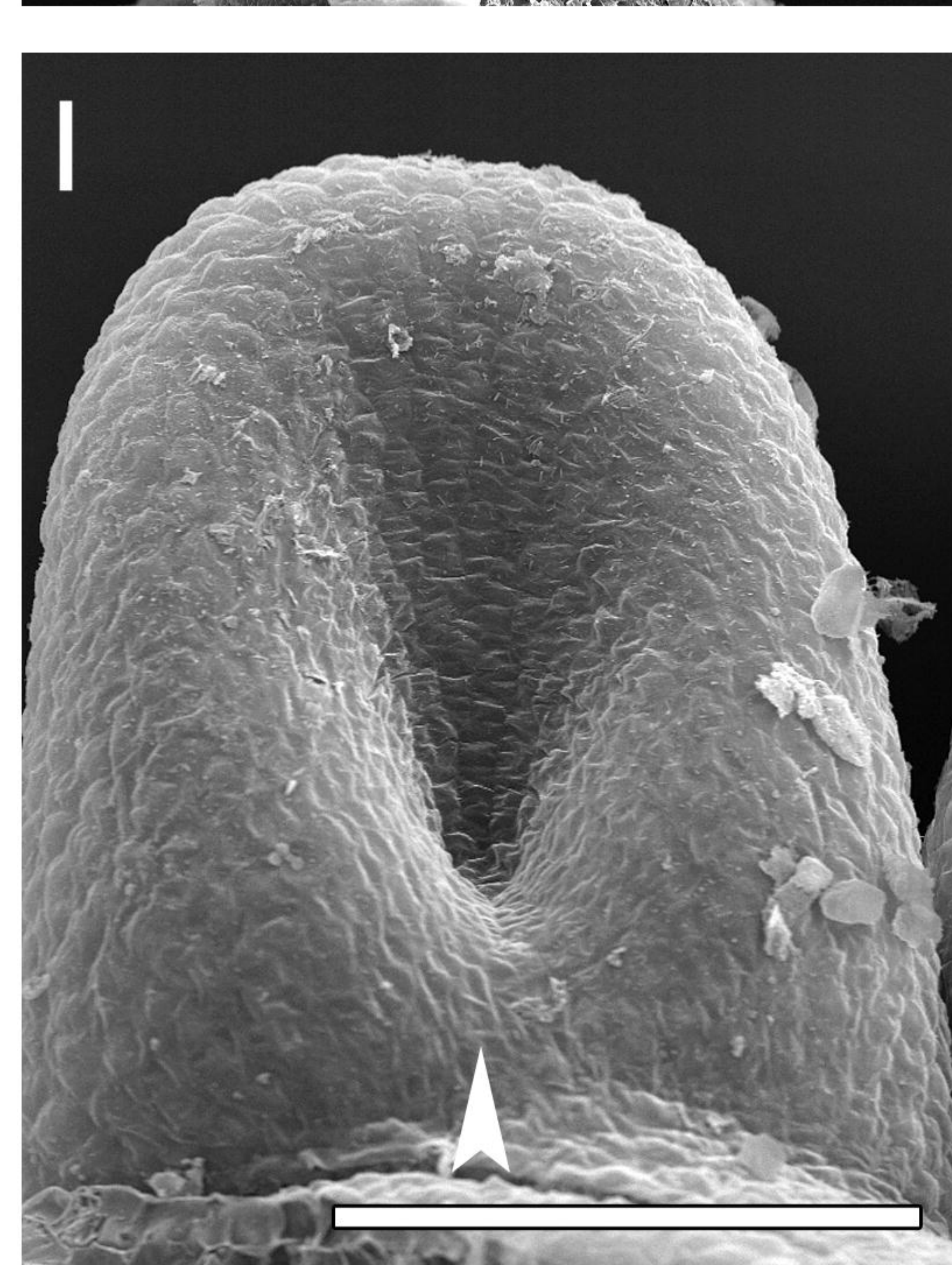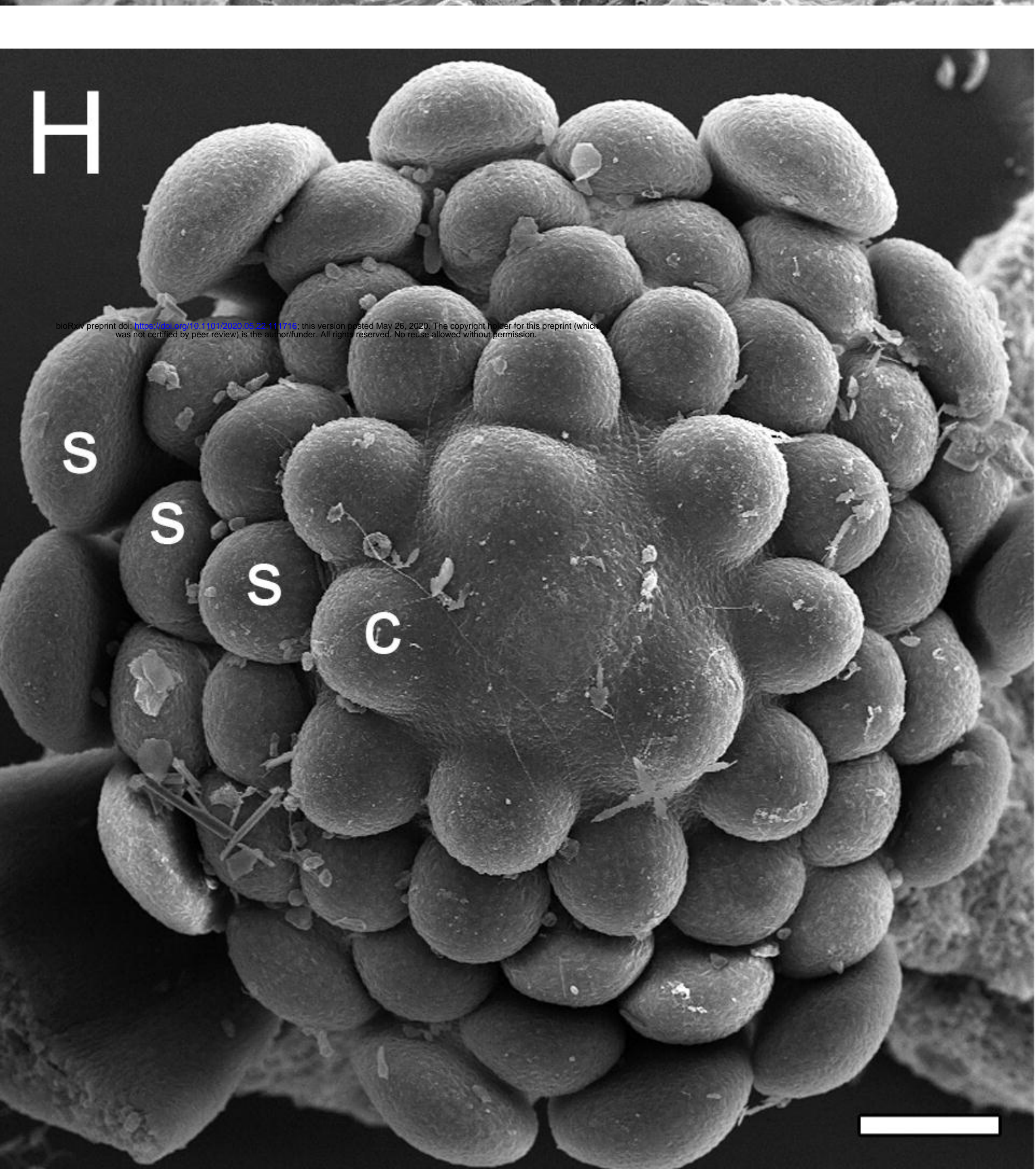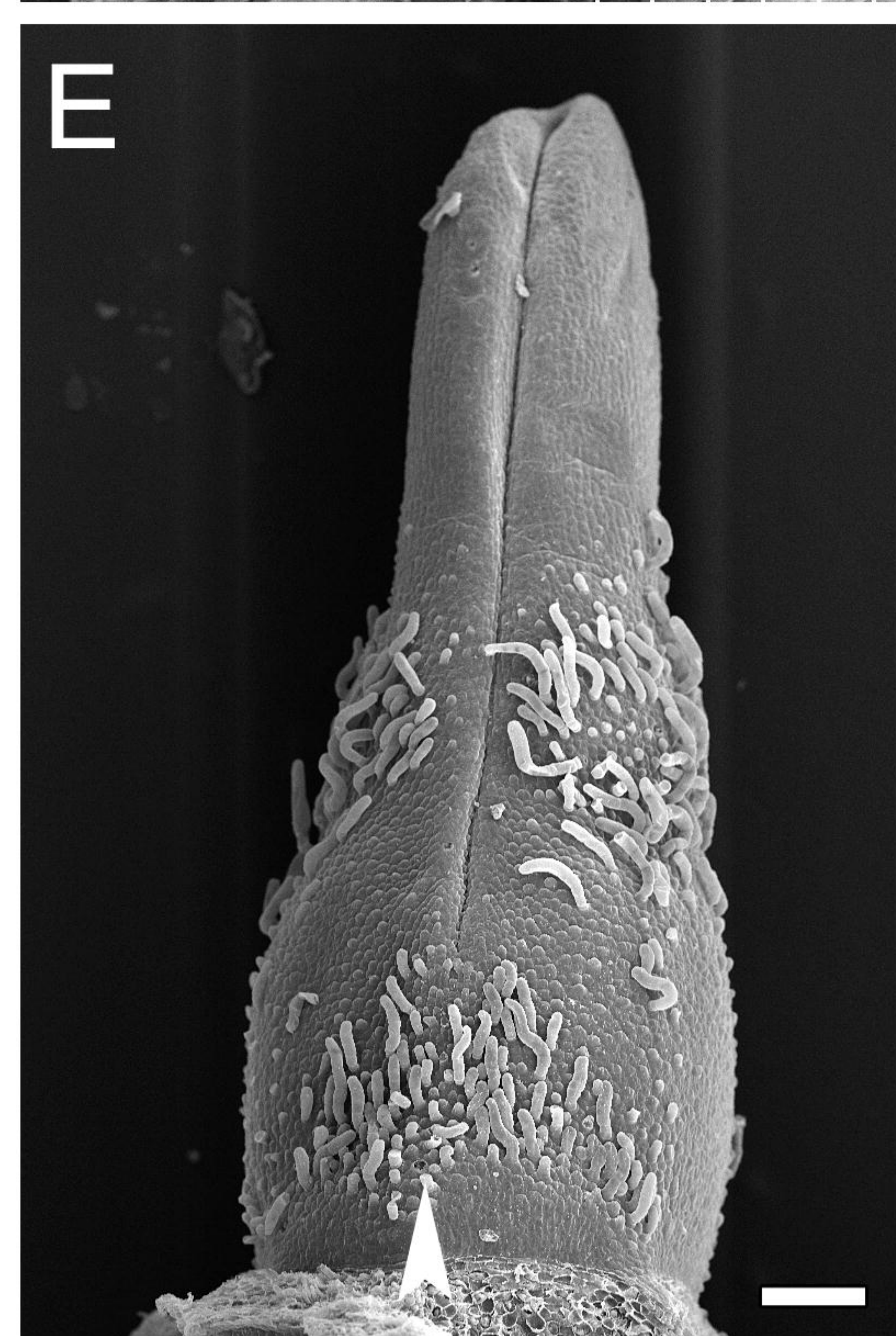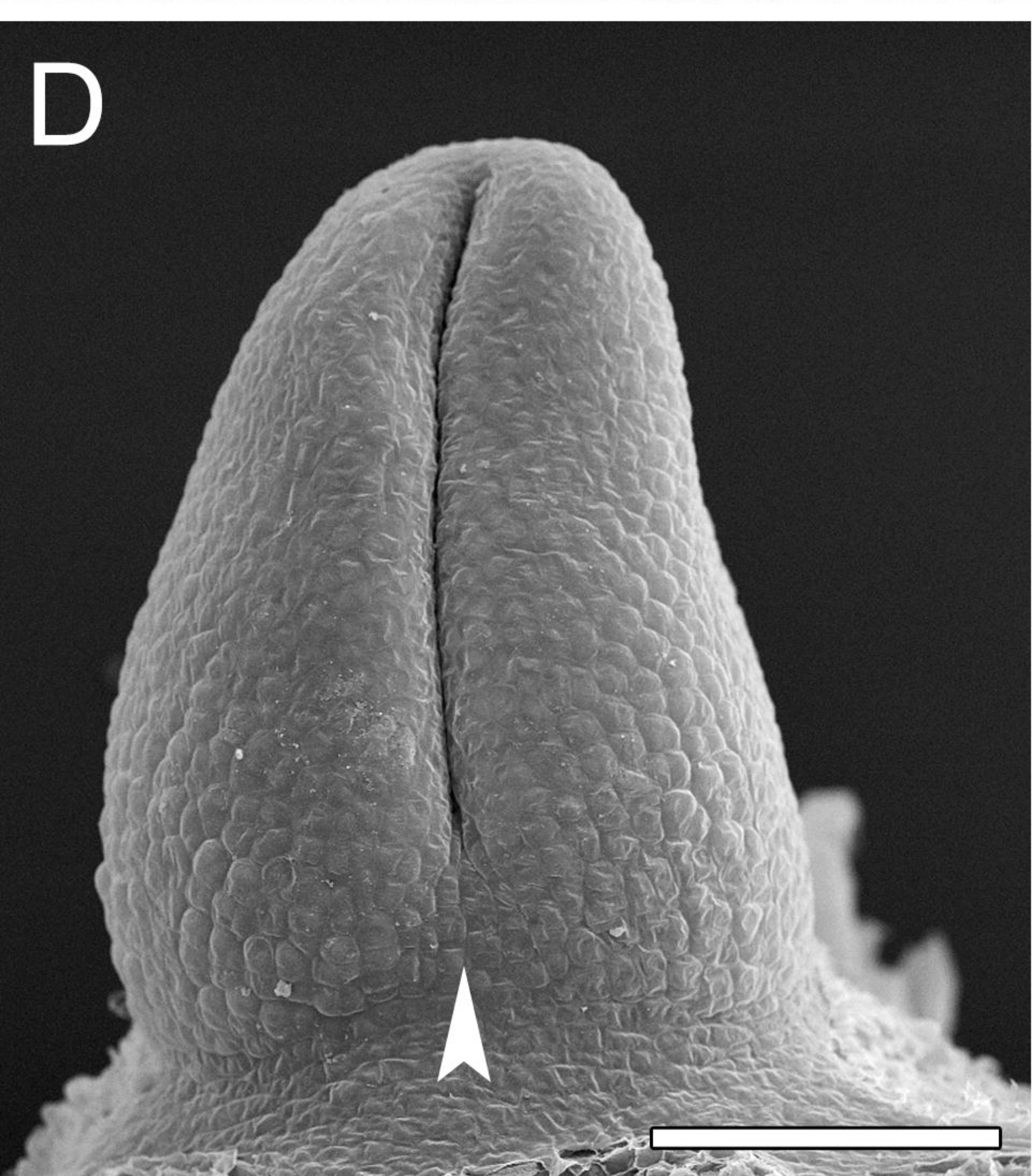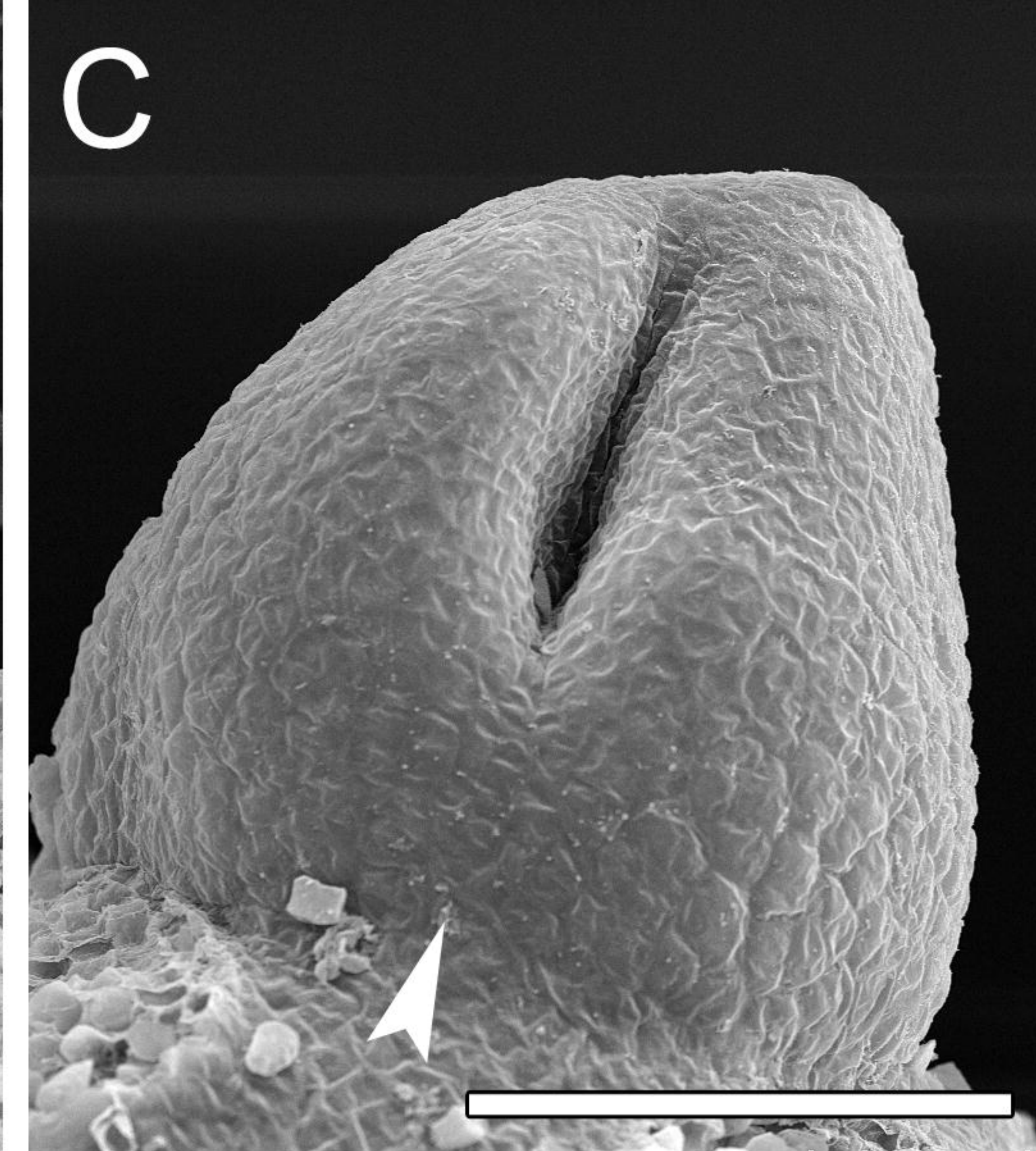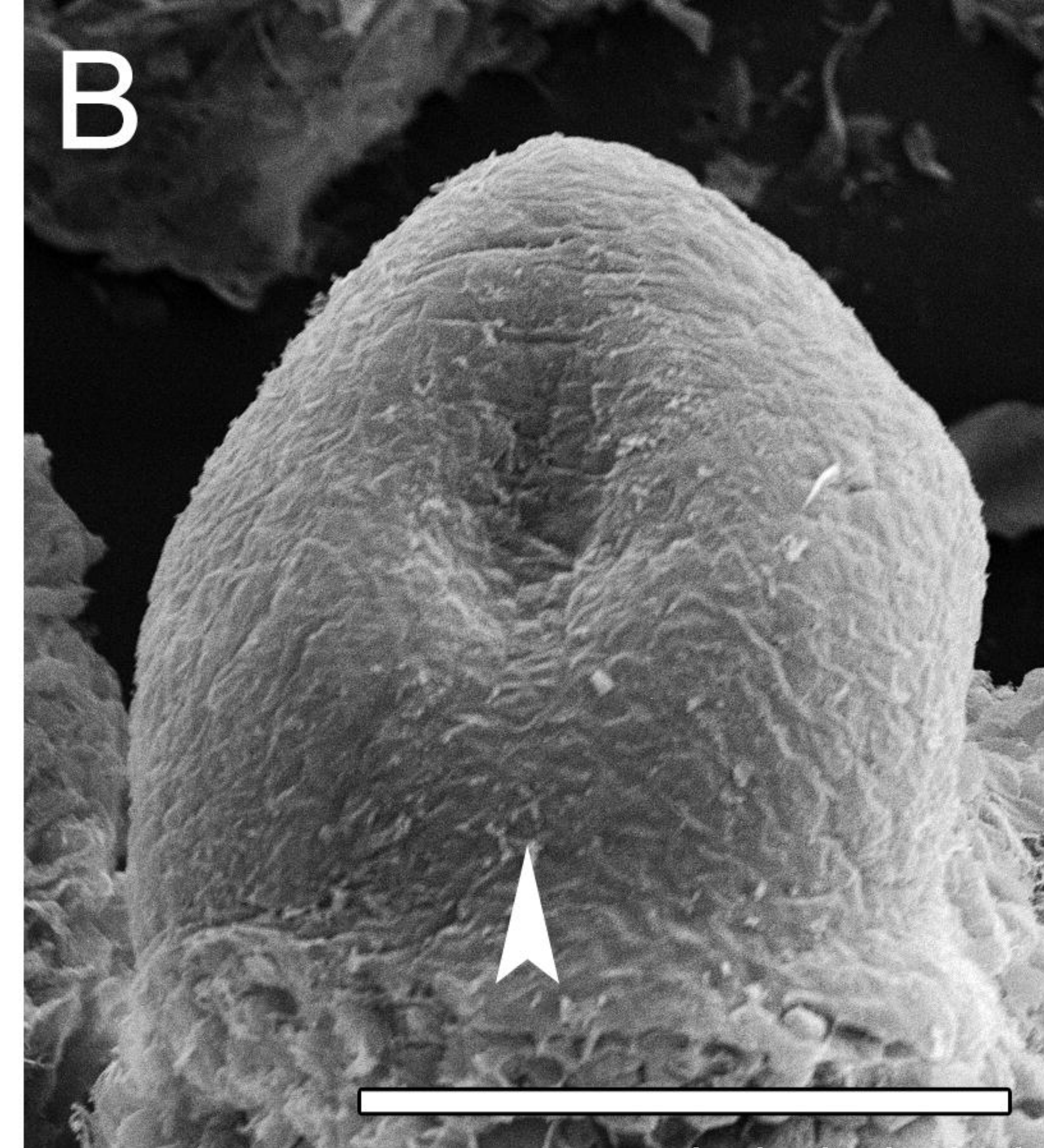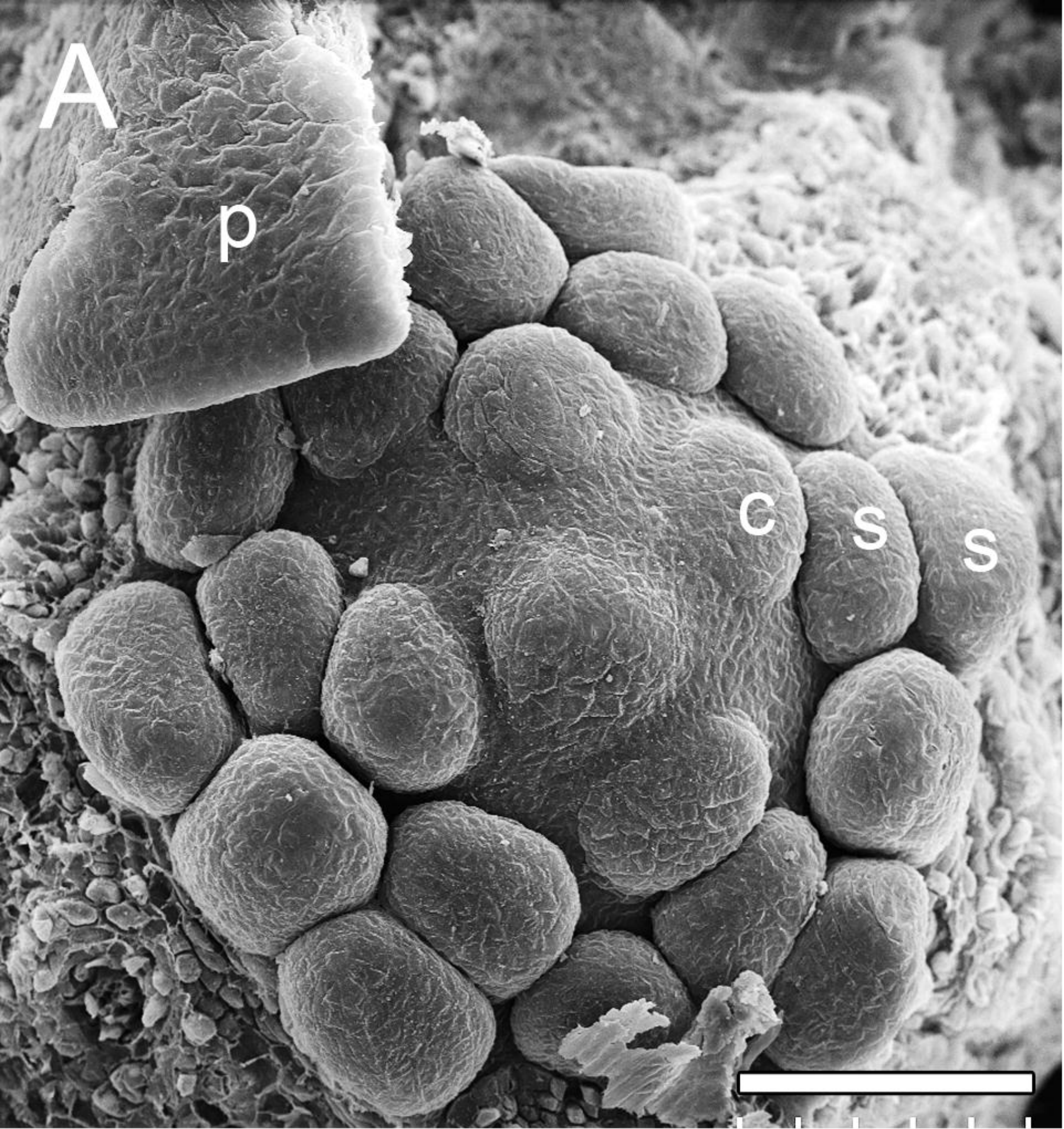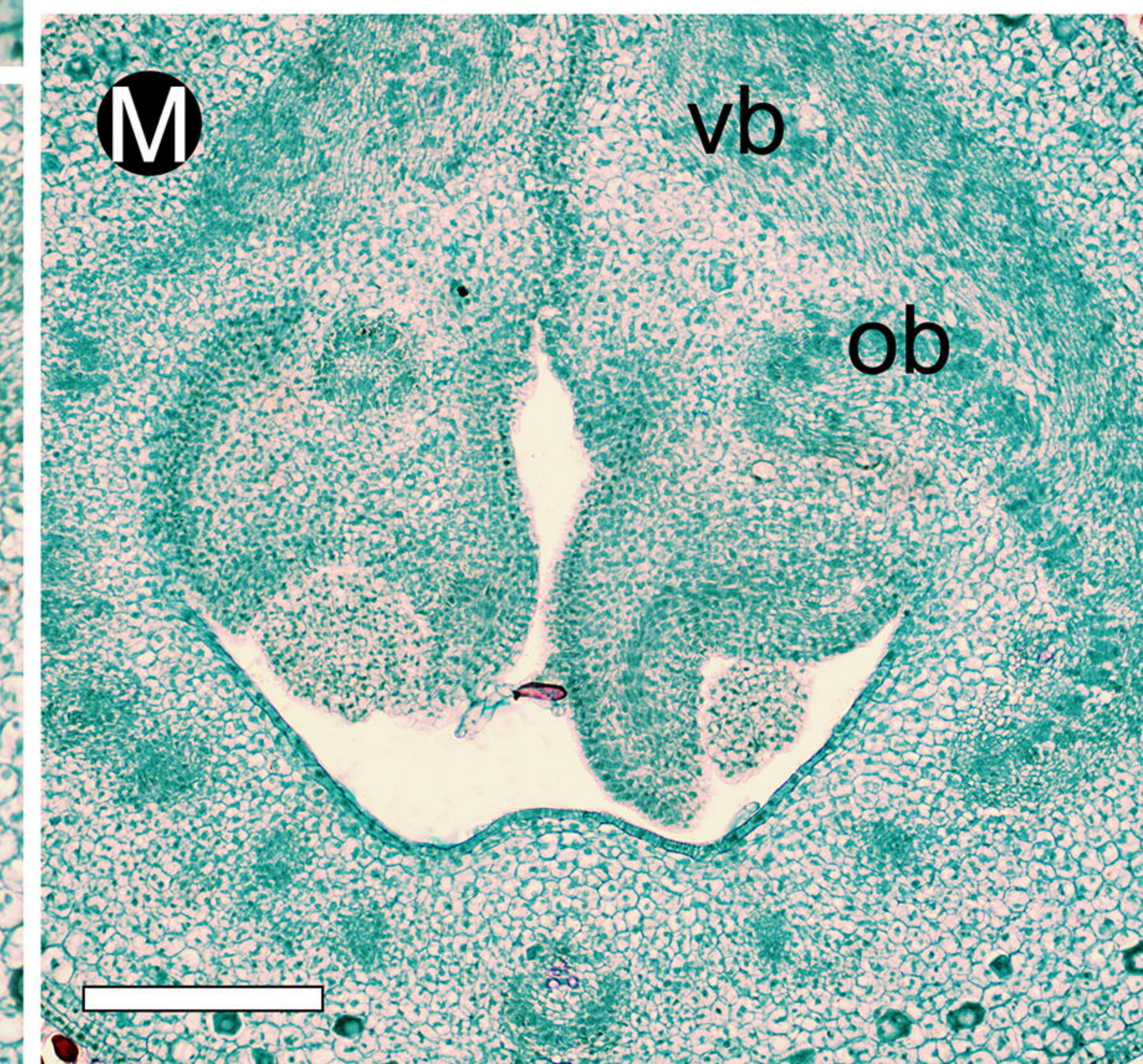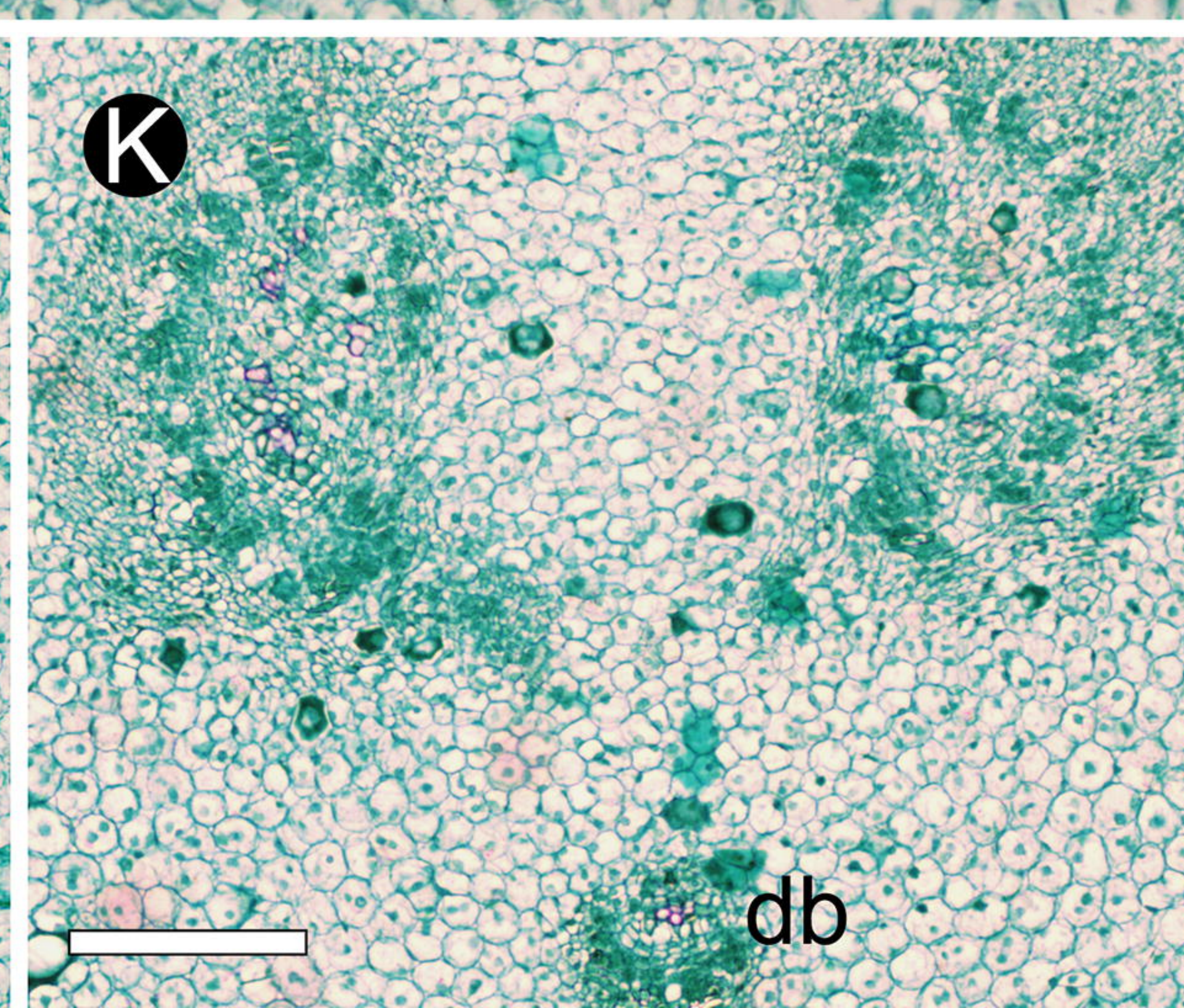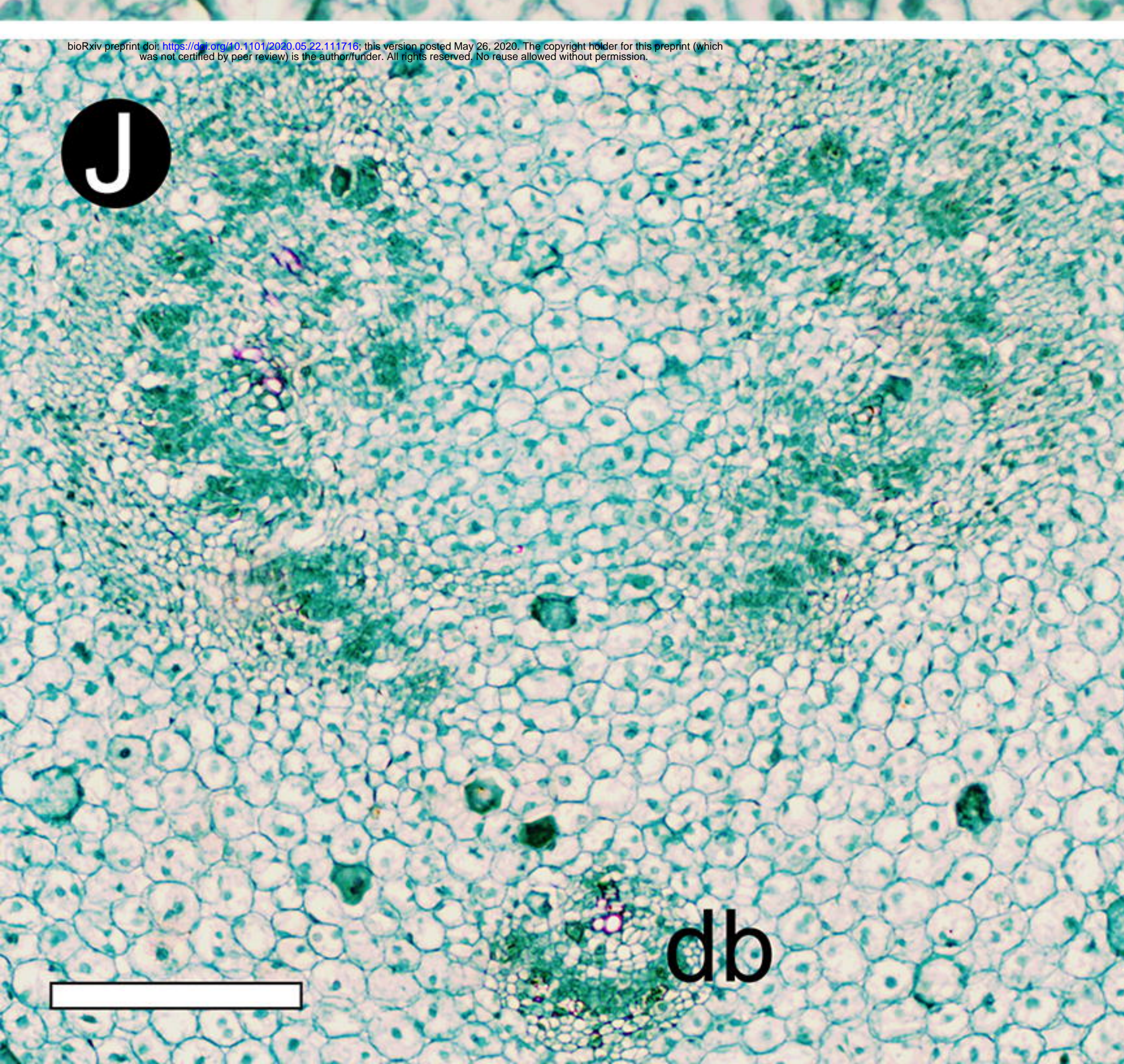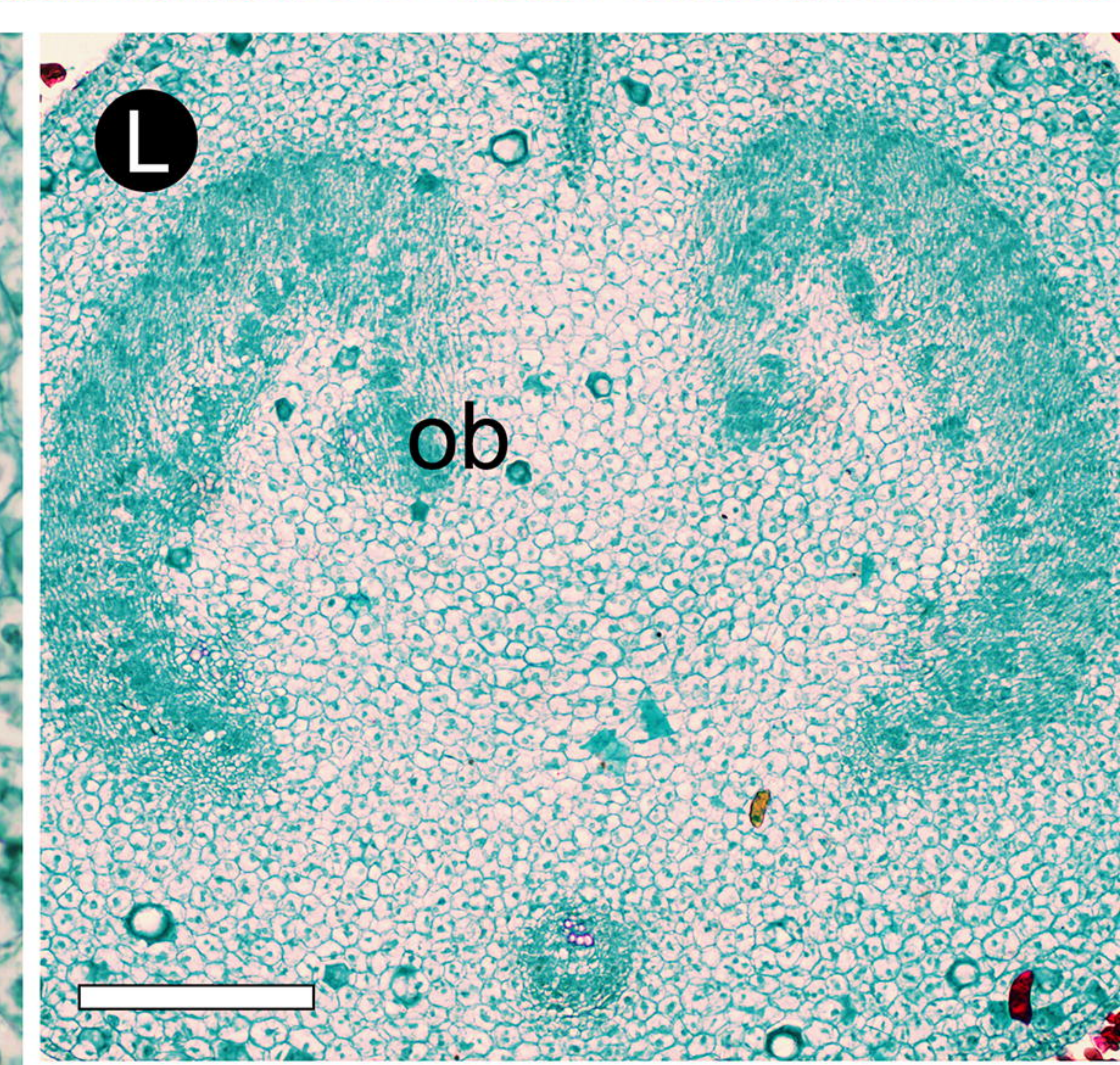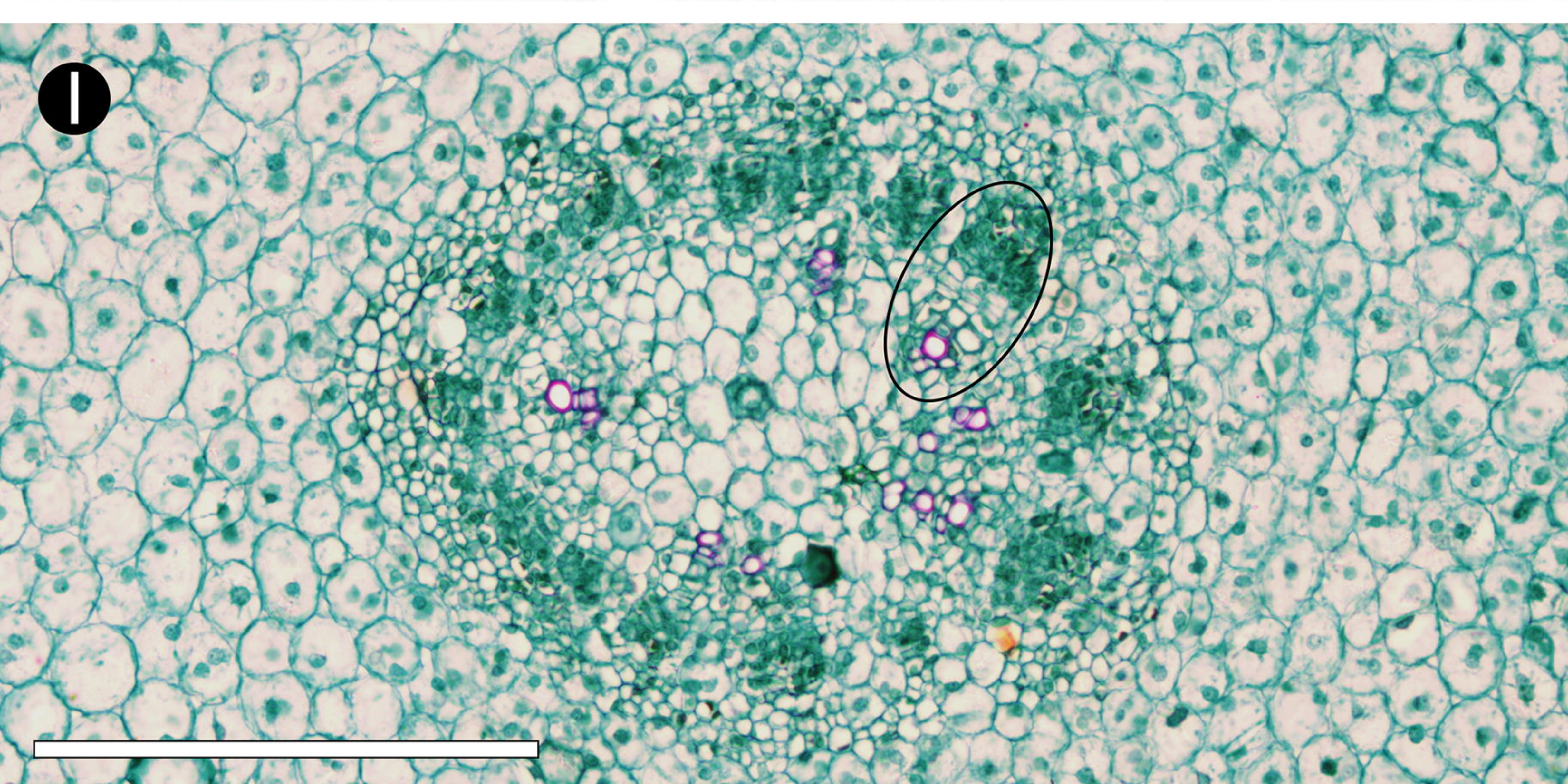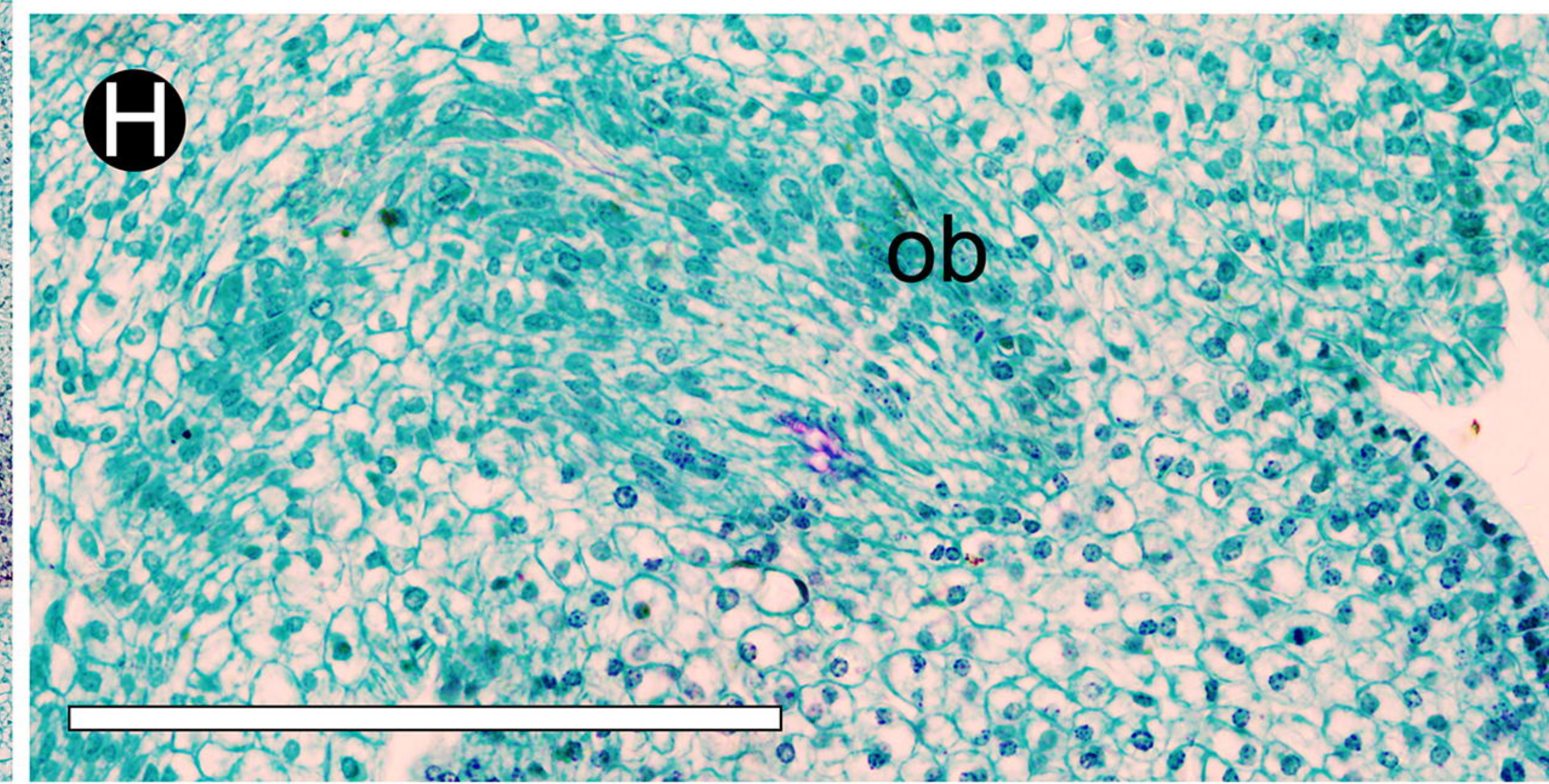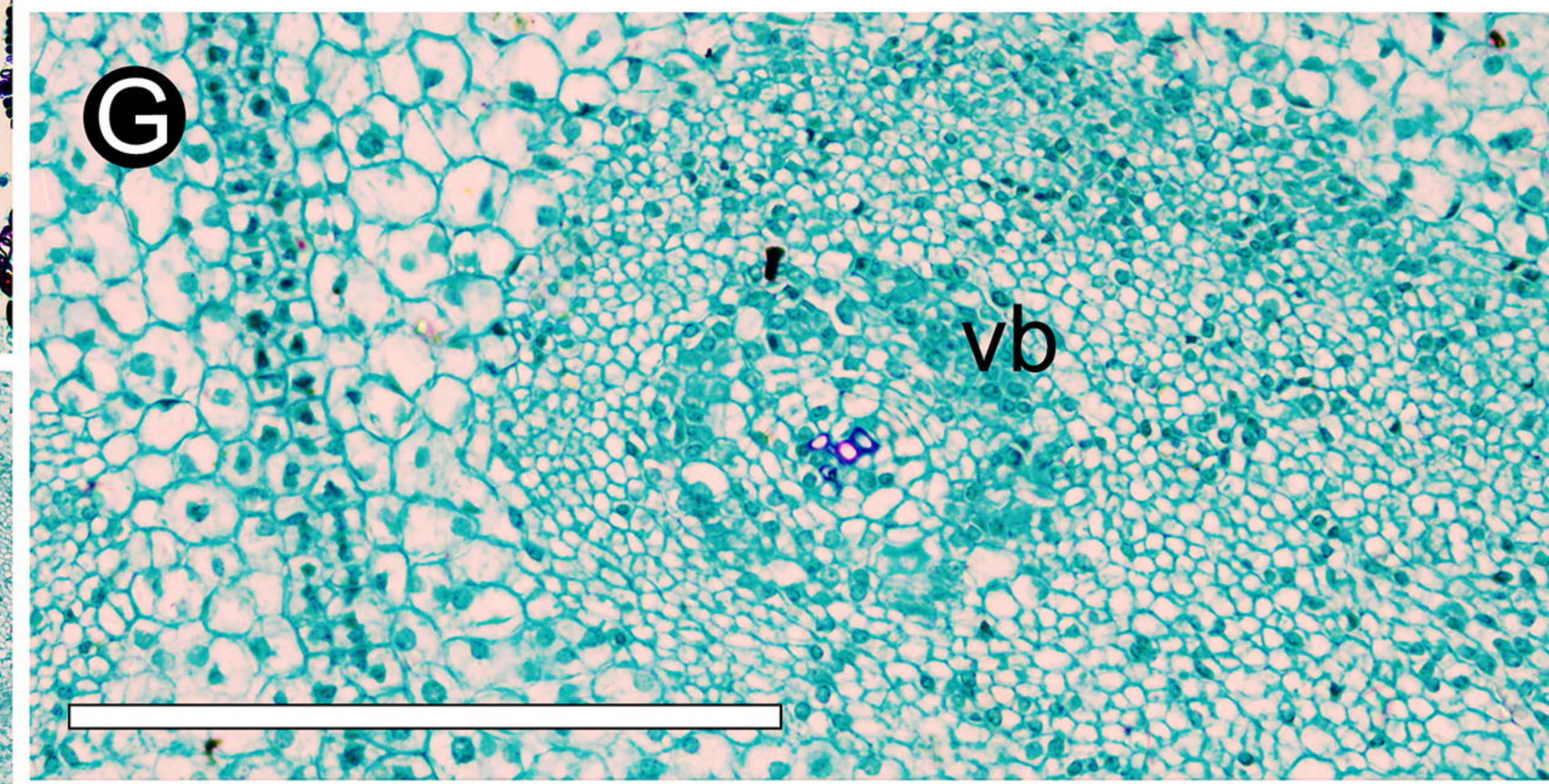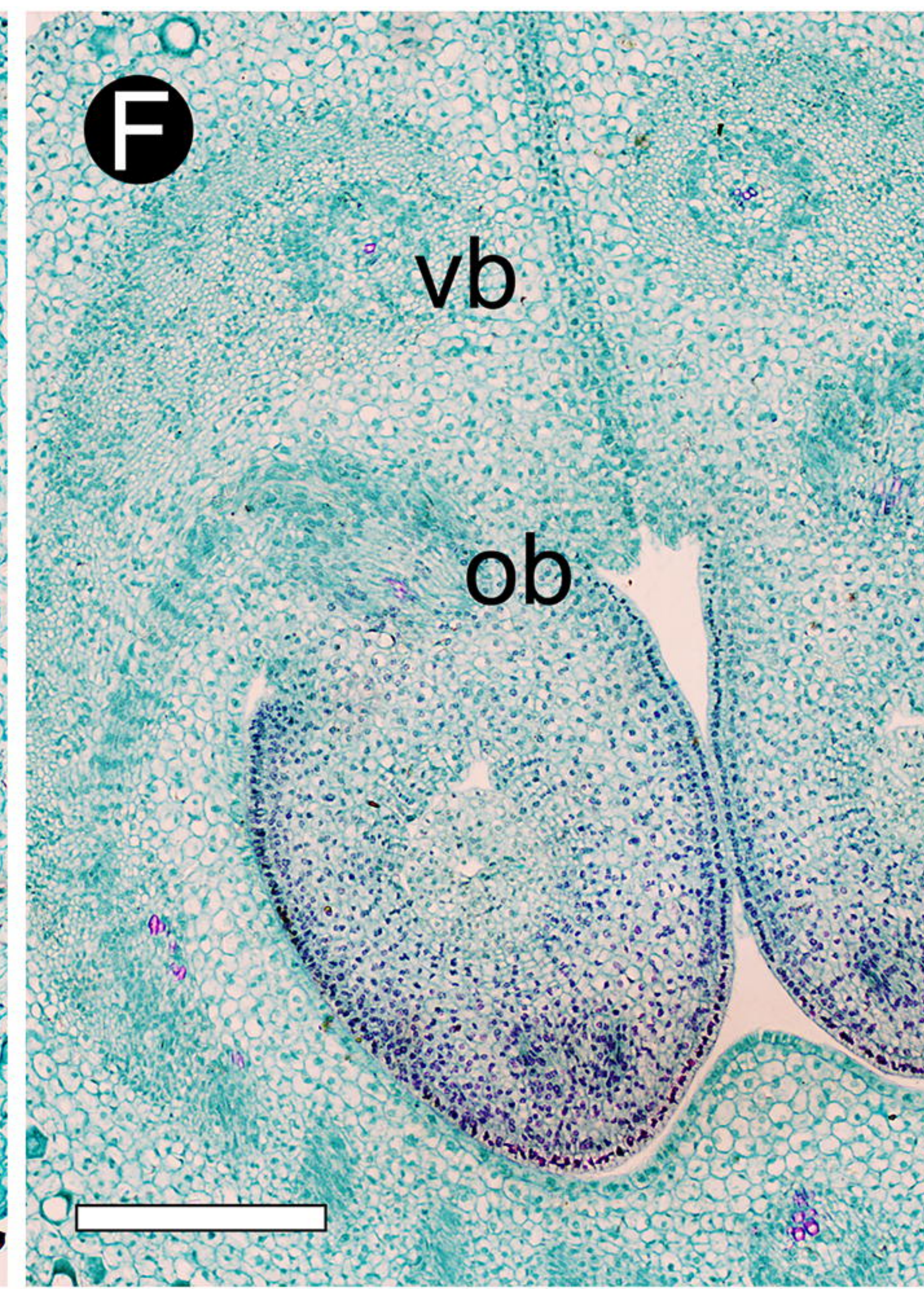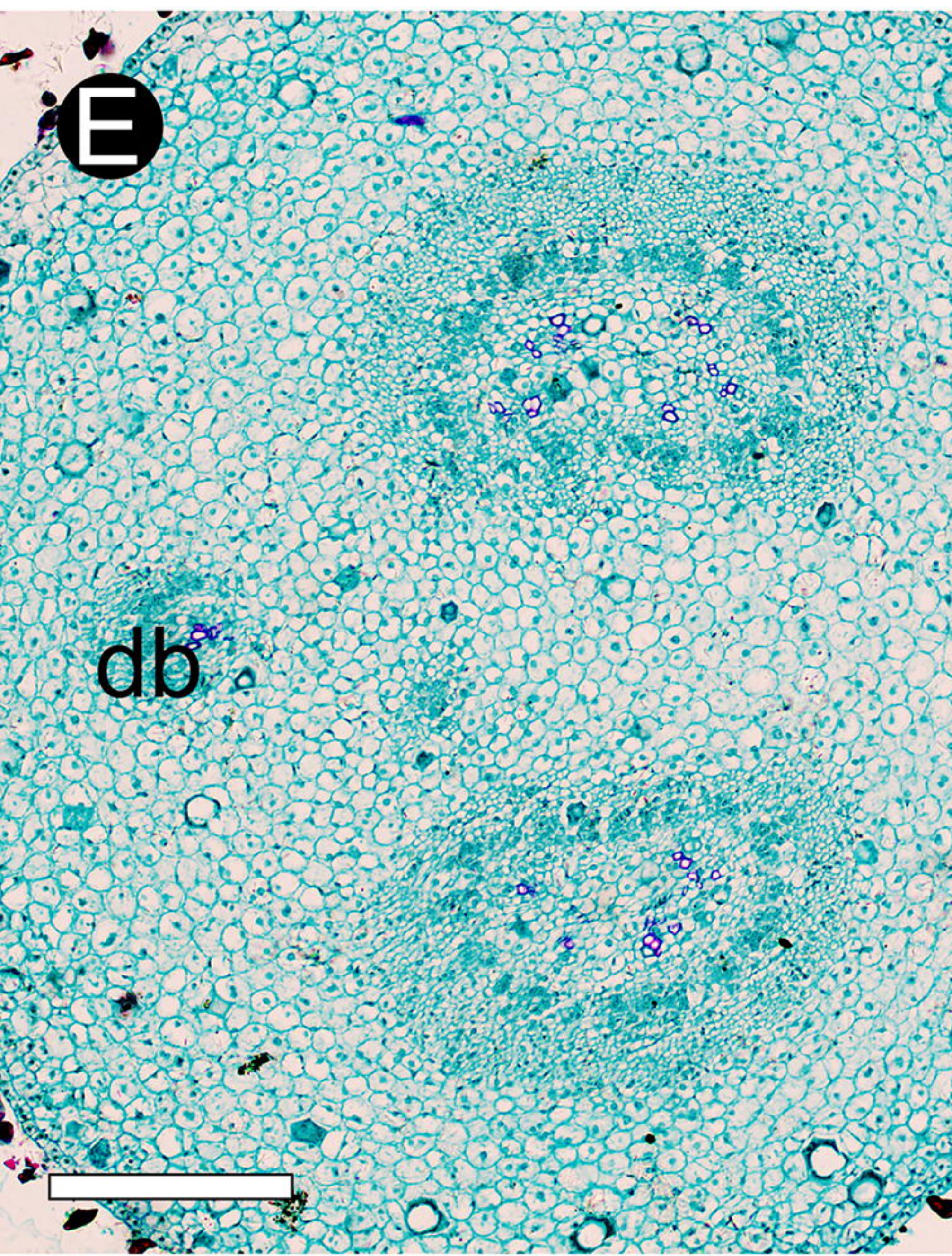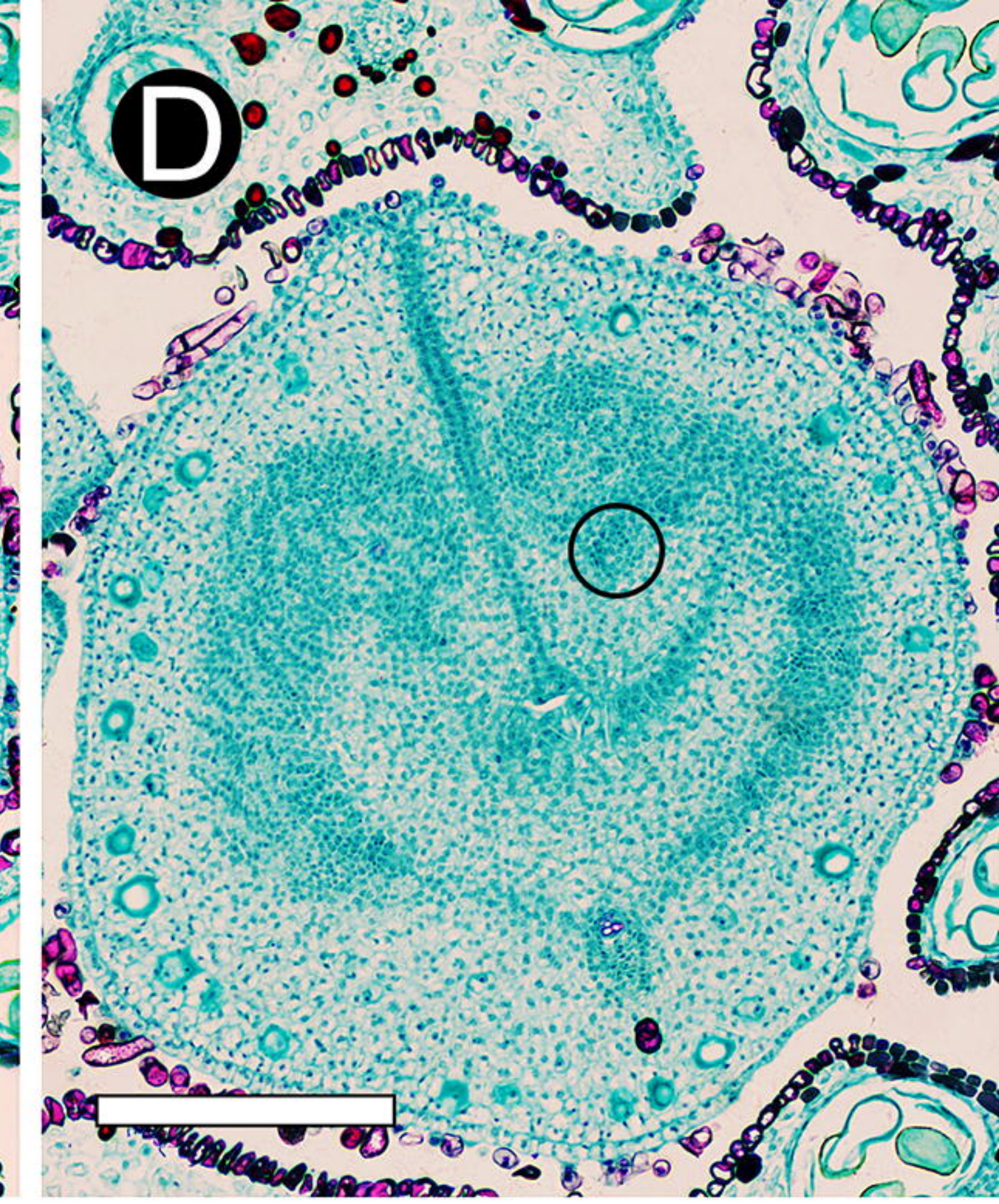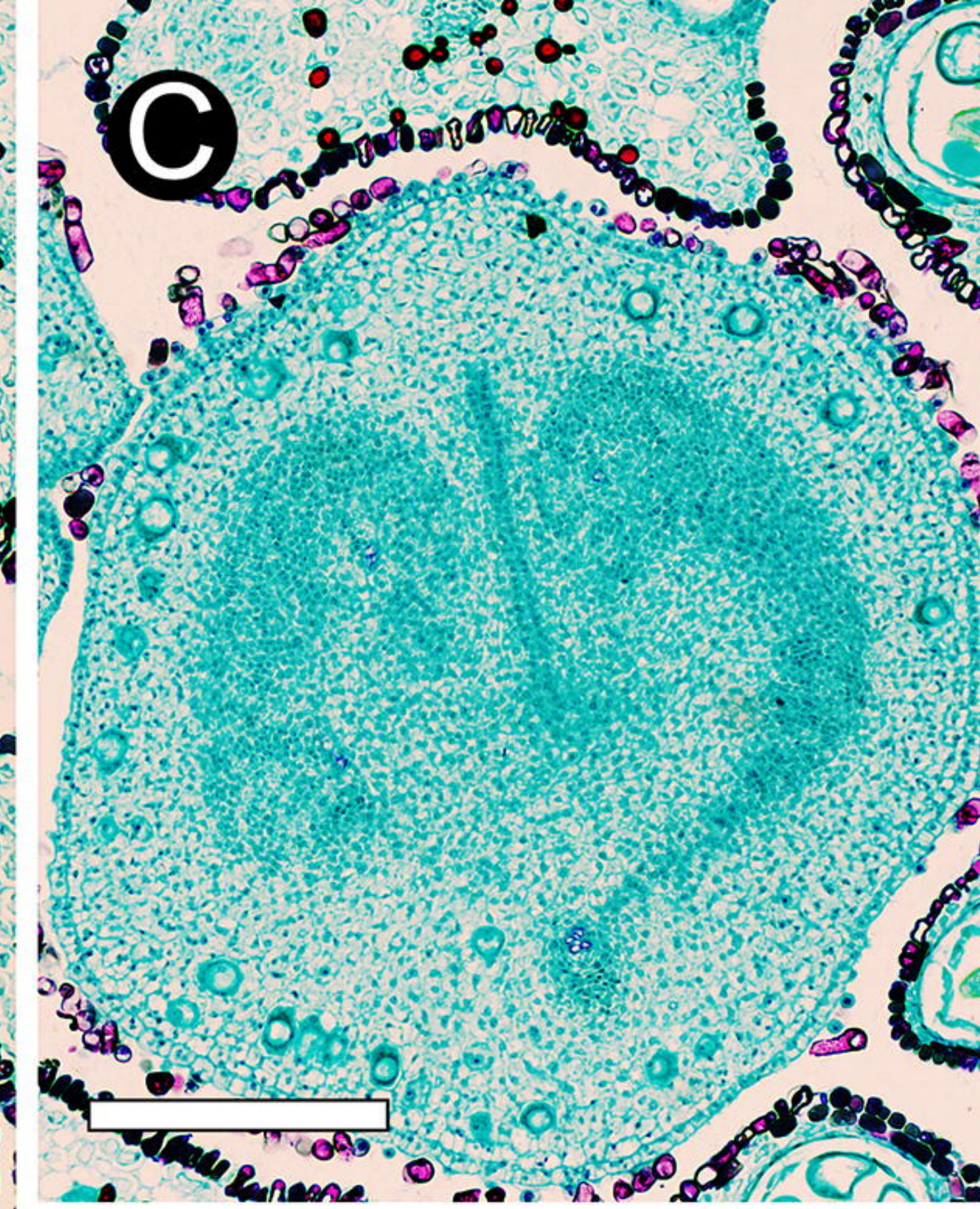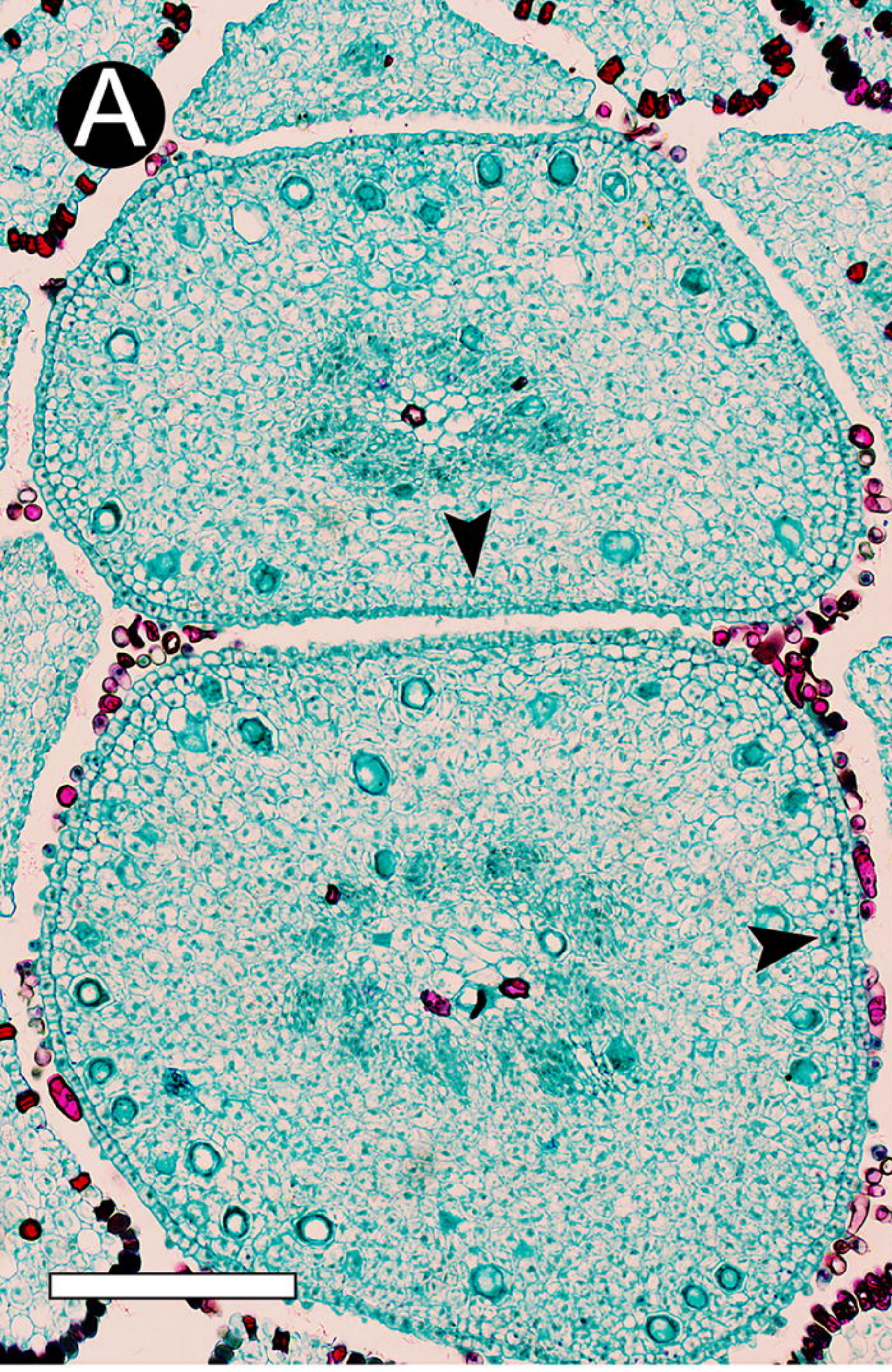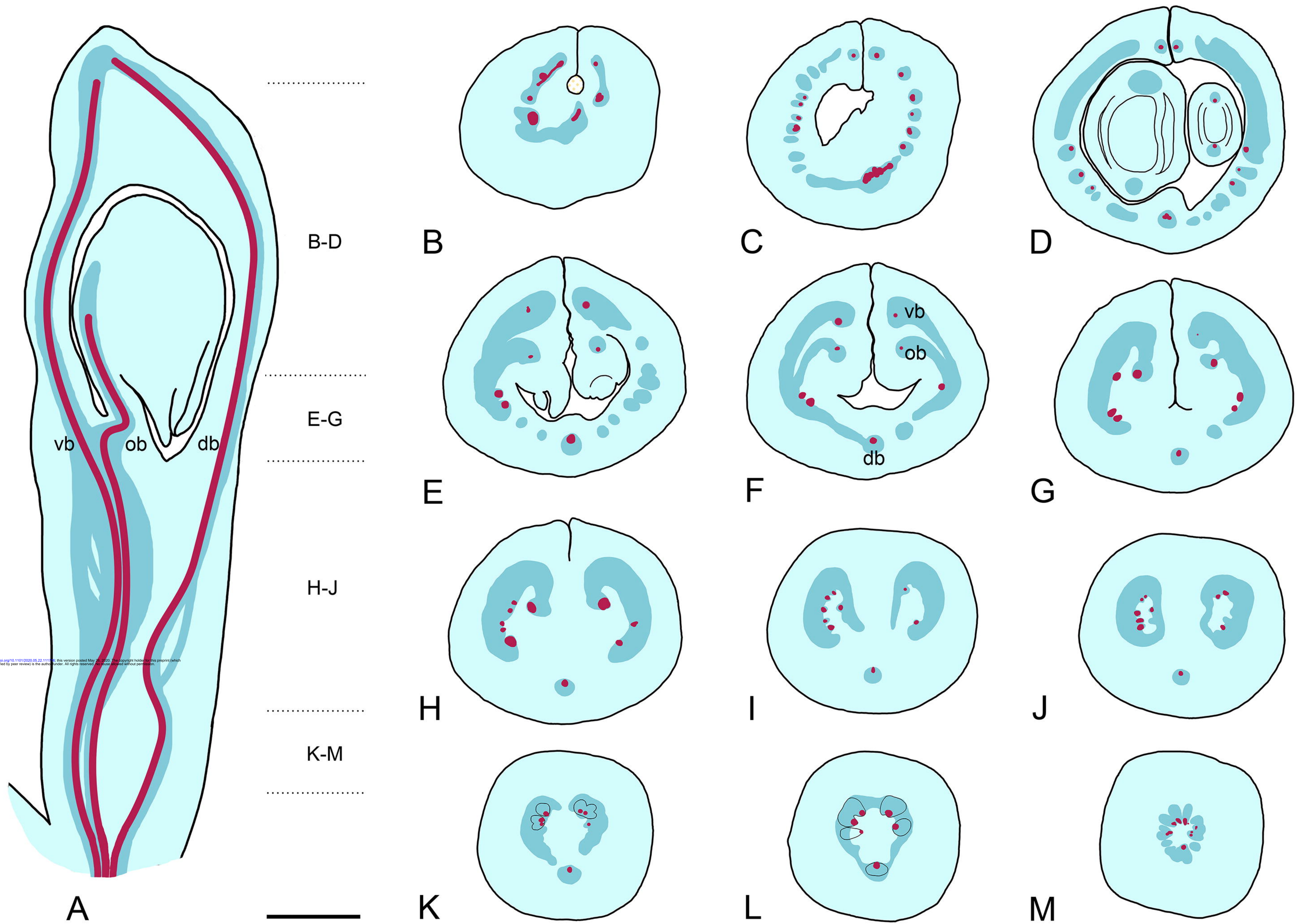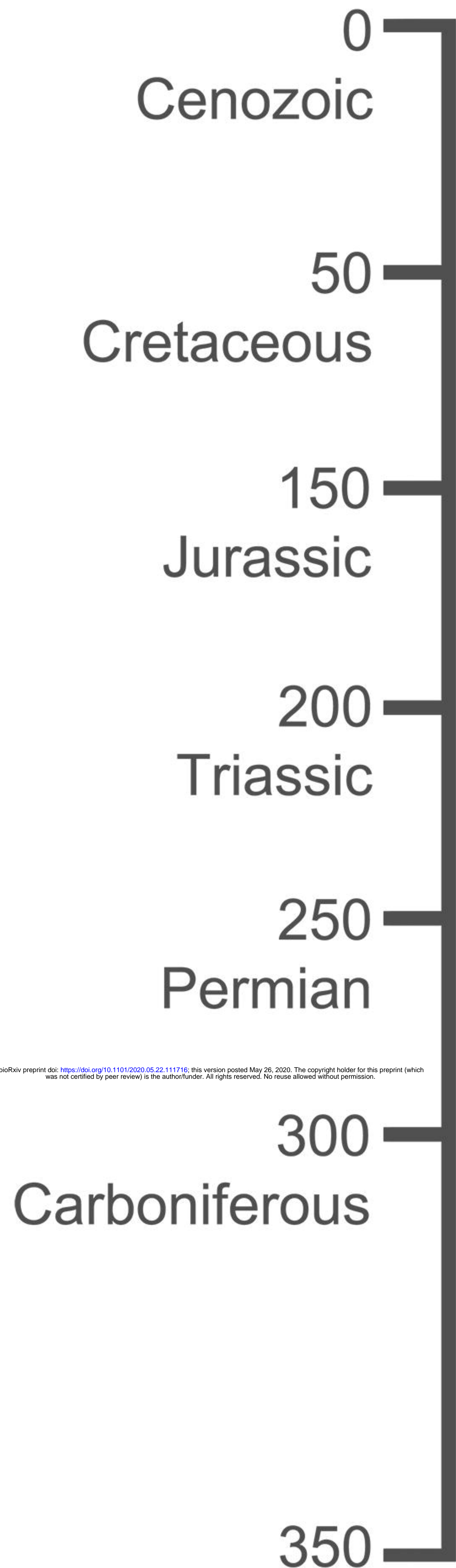
Conifers

Gnetophytes

Angiosperms

Ephedra

Anaxagorea

Cordaitales

ovule (with bracts)

shoot homologs

leaf homologs