

1 **Three-dimensional study of spur morphogenesis in the flower of**
2 ***Staphisagria picta* (Ranunculaceae) – from cellular level to organ scale**

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29

30 **Highlight**

31 A new method of 3D analysis of plant tissues at the cellular level revealed that spur
32 morphogenesis in *Staphisagria picta* is marked by an early phase of dominant cell
33 proliferation, followed by a phase of anisotropic cell expansion. Floral spur development is
34 analysed for the first time quantitatively, taking into account all tissues composing the organ,
35 namely epidermis and parenchyma.

36

37 **Abstract**

38 Floral spurs are invaginations borne by perianth organs (petals and/or sepals) that have
39 evolved repeatedly in various angiosperm clades. They typically store nectar and can limit the
40 access of pollinators to this reward, resulting in pollination specialization that can lead to
41 speciation in both pollinator and plant lineages.

42 Despite the ecological and evolutionary importance of nectar spurs, the cellular mechanisms
43 involved during spur development have only been described in detail in a handful of species,
44 primarily with respect to epidermal cells. These studies show that the mechanisms involved
45 are taxon-specific.

46 Using confocal microscopy and automated 3D image analysis, we studied spur
47 morphogenesis in *Staphisagria picta* (Ranunculaceae) and showed that the process is marked
48 by an early phase of dominant cell proliferation, followed by a phase of anisotropic
49 (directional) cell expansion.

50 The comparison with *Aquilegia*, another taxon of Ranunculaceae with spurred petals,
51 revealed that the convergence in form between the spurs of both taxa is obtained by partially
52 similar developmental processes. The analytical pipeline designed here is an efficient method
53 to visualize in 3D each cell of a developing organ, paving the way for future comparative
54 studies of organ morphogenesis in multicellular eukaryotes.

55

56 **Keywords:** 3D image analysis – Cells – Delphinieae – Floral spur – Morphogenesis – Petal
57 development – *Staphisagria*

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59

60 Introduction

61 The way in which similar forms or functions may be acquired independently through different
62 or similar developmental processes, involving homologous genes or completely different
63 genetic mechanisms is an important question in evolutionary biology. Floral nectar spurs are
64 invaginations of various dimensions borne on perianth organs. They have evolved repeatedly
65 in angiosperms, in various clades, on petals (for example in some lineages of orchids and
66 Lamiales, in *Viola* (Violaceae, Malpighiales), or *Valeriana* (Caprifoliaceae, Dipsacales)) or
67 sepals (for example in *Tropaeolum* (Tropaeolaceae, Dipsacales) (Ronse De Craene and
68 Smets, 1995)) (Figure 1). They usually store nectar, and may restrict access to this reward,
69 filtering the most efficient pollinators. Therefore their presence could be linked to
70 specialization in pollination and possibly lead to speciation in both pollinator and plant
71 lineages (Whittall and Hodges, 2007). Despite the ecological and evolutionary importance of
72 floral nectar spurs, the cellular mechanisms taking place during spur development have been
73 described into detail only in a few species, often considering only epidermal cells.
74 Development classically consists of two phases (cell proliferation and cell expansion and
75 differentiation which in plants are most generally segregated (Walcher-Chevillet and Kramer,
76 2016)), whose relative proportion and timing differ among clades. In *Aquilegia*
77 (Ranunculaceae, Ranunculales), detailed observations of the spur epidermis suggested that the
78 shape of the mature spur results from a combination of cell proliferation at early stages of
79 development, followed by anisotropic cell expansion allowing spur elongation. The
80 comparative study of spur development in four *Aquilegia* species revealed that anisotropic
81 cell expansion accounts for the differences in spur size among species (Puzey *et al.*, 2012). A
82 similar study conducted in *Linaria* (Plantaginaceae, Lamiales) suggested that differences in
83 spur length are better explained in this genus by differences in the number of cells resulting
84 from the initial phase of cell proliferation (Cullen *et al.*, 2018). The variation in the duration
85 of the phase of cell division supports the hypothesis that changes in the activity of cell cycle
86 genes and their regulators may be involved in the evolution of the nectar spur shape and
87 dimension. Like for *Aquilegia*, *Valeriana rubra* (basonym of *Centranthus ruber*,
88 Caprifoliaceae, Dipsacales), anisotropic cell expansion of epidermal cells plays a predominant
89 role in spur development (Puzey *et al.*, 2012; Mack and Davis, 2015).

90 The Ranunculaceae family comprises approximately 55 genera - ca. 2,500 species - that
91 display a great floral diversity, particularly at the perianth level, which may be composed of
92 both sepals and petals, or only sepals (reviewed in (Carrive *et al.*, 2020)). Spurs evolved
93 repeatedly in Ranunculaceae: twice on petals (in the stem lineages of the genus *Aquilegia* and
94 of the tribe Delphinieae) (Figure 2), and twice on sepals (in the stem lineages of the tribe
95 Delphinieae and of the genus *Myosurus*) (Hiepko, 1965; Kosuge and Tamura, 1988; Kosuge,
96 1994; Hodges, 1997; Erbar *et al.*, 1999; Endress and Matthews, 2006; Delpeuch *et al.*, 2022).
97 The diversity in spur shape observed in the genus *Aquilegia* makes this genus an ideal model
98 to study how the interactions with pollinators may have shaped floral morphology (Whittall
99 and Hodges, 2007), and also to identify the mechanisms of spur development and the genes
100 possibly involved (Ballerini *et al.*, 2019, 2020; Zhang *et al.*, 2020b). Spur morphogenesis
101 begins with the formation of a depression in the center of each petal primordium, that further
102 expands to form a hollow and narrow invagination (Tucker and Hodges, 2005; Ren *et al.*,
103 2011). At the cellular scale, the formation of the depression involves a short period of
104 localized cell divisions that stop progressively from the petal tip to the the site of initiation,
105 then the deepening of the cup is achieved by anisotropic cell expansion (Puzey *et al.*, 2012).
106 Cell proliferation is controlled by hormone signalling, principally involving auxine response
107 genes (Yant *et al.*, 2015; Zhang *et al.*, 2020). In flowers of Delphinieae, the dorsalmost petals

108 are spurred and nectariferous. These petals are nested within the spurred sepal (Jabbour and
109 Renner, 2012b), and their morphology (Figure 2) and development have been extensively
110 studied (Jabbour *et al.*, 2009; Chartier *et al.*, 2016; Chen *et al.*, 2018; Jabbour *et al.*, 2021). It
111 is a case of synorganization, that is to say the intimate structural connection of two or more
112 neighbouring floral organs forming a functional system (*i.e.*, a hyperorgan) (Specht and
113 Bartlett, 2009; Jabbour *et al.*, 2021). The length of the inner spurs determines the range of
114 pollinators able to collect nectar. The genus *Staphisagria* is sister to the remaining
115 Delphinieae and includes two species, namely *S. picta* and *S. macrosperma* (Léotard, 2002;
116 Jabbour and Renner, 2011). This genus was used to describe the development and structure of
117 the nectariferous hyperorgan (Jabbour *et al.*, 2021). A depression is initiated at the
118 primordium stage, deepens, and eventually forms a curved spur (Zalko *et al.*, 2021).
119 However, the cellular mechanisms involved - in terms of localization and duration of cell
120 proliferation and expansion - remain undescribed.

121 The aim of the present study is to finely explore spur morphogenesis by using the species *S.*
122 *picta* as a model to address the following questions: is spur length and curvature mostly
123 explained by cell proliferation or by cell expansion? Does the independent dual origin of
124 spurred petals in Ranunculaceae result from a convergence in shape or also from a
125 convergence in the pattern of cellular processes? Because of the phylogenetic affinity of
126 *Staphisagria* with *Aquilegia* (both genera belong to the same family), we expect anisotropic
127 cell expansion to account mostly for spur growth, cell division being restricted to the earliest
128 developmental stages. To test this hypothesis, we relied on the MorphoLibJ library, available
129 on the open-source platform for biological-image analysis Fiji (Schindelin *et al.*, 2012;
130 Legland *et al.*, 2016), a collection of generic tools dedicated to the analysis of plant cells on
131 3D images. We characterized spur morphogenesis in *S. picta* using confocal microscopy and
132 described the three-dimensional characteristics of cells (including epidermis and parenchyma)
133 at successive developmental stages. The results are compared with those previously obtained
134 in *Aquilegia* (Puzey *et al.*, 2012), *Centranthus* (Mack and Davis, 2015), and *Linaria* (Cullen
135 *et al.*, 2018), focused on epidermal cells.

136

137 **Materials and methods**

138 *Plant material*

139 Seeds were obtained from the French National Museum of Natural History (MNHN).
140 Reference herbarium specimens corresponding to plants grown from the same set of seeds are
141 kept at P herbarium (MNHN) (barcode P04023155,
142 <http://coldb.mnhn.fr/catalognumber/mnhn/p/p04023155>, and P04023156,
143 <http://coldb.mnhn.fr/catalognumber/mnhn/p/p04023156>). Floral buds were sampled from
144 plants grown at the Jardin Botanique de Launay (Orsay, France) in September 2021, and
145 fixed in FAA (90% alcohol 70%, 5% formaldehyde, 5% acetic acid). The corolla of
146 *Staphisagria* species comprises four fully-developed petals. At anthesis, the two lateral petals
147 are flat, whereas the two dorsalmost petals are spurred (Figure 2) and nested within the
148 hollow sepal. The nectariferous tissue is located all along the inner epidermis of the spur
149 (Zalko *et al.*, 2021). Floral buds of *Staphisagria picta* were collected at 10 successive
150 developmental stages, from stage 01 (bud sampled after the resumption of development, the
151 petal is flat) to stage 10 (mature petal). The development of reproductive organs was used as
152 a reference to calibrate the developmental sequence (Supplementary Fig.S1, adapted from

153 (Delpeuch *et al.*, 2022)). Buds representative of each of the ten developmental stages,
154 covering the whole developmental sequence, were selected (one bud per stage), and one
155 dorsal spurred petal – left or right – was dissected for further analysis (Figure 3).

156 *Confocal microscopy*

157 Tissues were treated as described by Schaefer *et al.* (2017). Cell walls were stained with
158 fluorescent brightener 28 as described by Belcram *et al.* (2022) with minor modifications as
159 follows. Samples fixed in FAA were transferred in ethanol 70%, and then progressively
160 rehydrated in 50% ethanol, followed by 10 minutes in 30% ethanol. They were incubated in 0.2
161 N sodium hydroxide/1% SDS for two hours at room temperature and rinsed in water. Samples
162 were then simultaneously cleared and stained by an incubation overnight in a clearing solution
163 (25% urea, 15% deoxycholate, 10% xylitol in distilled water) with the addition of 0.1%
164 fluorescent brightener 28 (the stock solution is a 1% solution with one drop of sodium
165 hydroxide 10 N to allow complete dissolution). Samples were rinsed in the clearing solution
166 without the staining solution; and mounted in Citifluor AF1 (Agar Scientific). The image
167 acquisitions were made with an inverted Zeiss Observer Z1 spectral confocal laser microscope
168 LSM 710 and with a 25x objective (LD LCI Plan-Apochromat 25x/0.8 Imm Korr DIC M27).
169 Fluorescence of the Fluorescent Brightener 28 dye was recorded using a 405-nm excitation and
170 a selective emission window of 410–485 nm. Each sample was imaged as a Z-stack (of
171 longitudinal sections) encompassing the entire thickness of the spur. We used a voxel size of
172 0.35 x 0.35 x 0.5 μm for the first five stages and 1.1070 x 1.1070 x 1.1 μm for the last five.

173 *Scanning electron microscopy*

174 Buds of *Staphisagria picta* at mature stage (stage 10) and between stages 06 and 07 were fixed
175 in FAA. They were placed in ethanol 70% the day before dissection. Petals were extracted and
176 longitudinal and tangential sections were made under a stereoscope (Nikon SMZ 745T). The
177 dissected petals were dehydrated in absolute alcohol and dried using an Emitech K850 critical-
178 point dryer (Quorum Technologies), mounted on aluminum stubs with colloidal graphite,
179 sputter-coated with platinum (60 s of metallization) using a JFC-1200 fine coater (JEOL), and
180 observed using an SU3500 scanning electron microscope (Hitachi).

181 *Imaging strategy*

182 Buds at early developmental stages (stages 01 to 05) were imaged as a whole, without
183 isolating the developing petals, because of their very small size. In larger buds (stages 06 to
184 10), petals were extracted, and the spur was isolated and tangentially sectioned. Each side was
185 imaged separately. The petals were oriented in space according to their insertion on the floral
186 receptacle. For the largest samples (stages 06 to 10), cells were first analysed along the entire
187 imaged spur. In a second step, the spur was divided into three sectors, corresponding
188 respectively to the proximal zone (sector P: closest to the receptacle), the median zone (sector
189 M), and the distal zone (sector D: tip), and the imaging and analyses were repeated for each
190 sector. The sectors were defined along the corresponding longitudinal axis. They were first
191 defined on the mature petal, and transferred to the other stages, adapting their size. Analyses
192 were performed on the upper side and lower side of the spurs of stages 06 to 10 separately, to
193 study the curvature of the spur in detail. The lower side was defined as the part of the spur
194 located in the prolongation of the zone of insertion to the floral receptacle, and the upper side
195 as the part opposite the zone of insertion to the floral receptacle (Figure 3).

196 *Segmentation processing*

197 Images were subdivided into smaller parts to fit in the hardware memory using the software
198 Fiji (Schindelin *et al.*, 2012). First, we applied on the images the pre-processing of PlantSeg
199 (Wolny *et al.*, 2020), a pipeline for volumetric segmentation of plant tissues into cells. This
200 pre-processing employs deep learning, in particular a convolutional neural network to predict
201 cell boundaries. Second, all images were blurred and segmented with the Morpholib package
202 (Legland *et al.*, 2016), selecting parameters “catchment basins method” with a tolerance of
203 0.5. Parameters of the segmentation were chosen empirically by performing manual
204 segmentations tests. Because of the size and number of acquired data, automation of the
205 segmentation process launched with a homemade Python script was necessary.

206 *Visualization and analysis of segmented petals*

207 A script written in Python language using the package “plotly” allowed us to visualize the
208 result of the segmentations. All segments were reassembled using their coordinates and each
209 cell was displayed according to its x, y, and z coordinates. Petals were reconstructed in three
210 dimensions and the characteristics of cells (volume, sphericity, and orientation of aspheric
211 cells) were visualised using different colours, and further analysed quantitatively. Cell density
212 in each sector is then simply the inverse of the average cell volume. The volume of the organ
213 or sector is estimated by the total volume of the cells, independently of the hollow part of the
214 spur.

215 The data extracted from image analysis provide information on the alignment of cells along a
216 given axis, for example the spur longitudinal axis. The value of the angle $\theta \in \left[0, \frac{\pi}{2}\right]$ between
217 cell orientation vector and the axis was used as a measure of the alignment of the cells with the
218 axis. To interpret the result, the theoretical distribution of angles for an isotropic distribution of
219 direction is shown in black on the figures. This distribution $p(\theta)$ can be calculated by
220 considering that the surface element of the unit sphere spanning θ to $\theta + d\theta$ is $dS =$
221 $d\theta \sin(\theta) 2\pi$, thus $p(\theta) = C \sin(\theta)$, with C a constant to be determined. Because p is a
222 probability distribution we have $1 = \int_0^{\pi/2} p(\theta) d\theta = \int_0^{\pi/2} C \sin(\theta) d\theta = [-C \cos(\theta)]_0^{\pi/2} = C$,
223 i.e. $C = 1$ and $p(\theta) = \sin(\theta)$.

224 *Post-processing*

225 Pre-processing with PlantSeg amplifies the noise of the acquisition in the background. As a
226 result, “false cells” may appear when performing the segmentation. To discard most of these
227 “false cells”, the background was removed by applying a mask on each petal. Such mask was
228 obtained by running segmentation using CLAHE instead of PlantSeg, which is less precise but
229 has the advantage of being less prone to generating “false cells” in the background. To create
230 the 3D mask, the volume was partitioned into smaller cubes, the number of cells in each cube
231 was calculated, and cubes with a number of cells under a threshold empirically chosen were
232 masked. This process allowed us to discard regions outside the petal without discarding regions
233 of the petal itself. The mask was then applied on the predictions of the PlantSeg segmentation
234 to remove “false cells”.

235 Cell outliers, i.e. the 5% largest and smallest cells in terms of volume, were filtered out. To
236 visualize the interior of the petals, we relied on the opacity of the dots or on virtual sections.

237 The code that allowed the automation of the segmentations and the visualization of the data is
238 available on github [<https://github.com/paulinedlpch/morphogenesis>].

239

240 **Results**

241 *Stages of floral development*

242 Flower development of *Staphisagria picta* begins with the initiation of the five sepal
243 primordia, followed by petal initiation. Petal development is arrested shortly after stamen
244 initiation. It resumes after the initiation of carpel primordia. In the present study, we
245 examined only the developmental stages that take place after the developmental stasis of
246 petals. At stages 01 and 02, the petal is flat, thick, and curved, slightly lobed in the distal part.
247 A depression in the blade appears between stages 02 and 03, in the shape of a broad pocket.
248 The depression deepens, developing into a spur between stages 05 and 07. Between stages 07
249 and 08, the spur becomes slightly curved in its proximal part (close to the receptacle). The
250 rest of the spur becomes curved between stages 08 and 09. The spur lengthens throughout the
251 whole development. At stage 10, the mature spur is curved, *ca.* 7.5 mm long and 2 mm wide
252 at the opening.

253 *Cellular characteristics during petal development*

254 On raw images obtained by confocal microscopy and 3D visualizations, epidermal cells
255 appeared larger and had a more regular shape than inner cells (Figure 4AB). The inner
256 epidermal layers are evenly organized. Spherical, small and disordered cells could be
257 distinguished at stage 01 at the position of the future lobes. From stage 02, the central zone
258 presents evenly ordered and slightly elongated cells. Large and irregular cells were observed
259 towards the insertion point of the petal. From stage 03, three rows of elongated cells oriented
260 along the longitudinal spur axis were observed, which could correspond to the vasculature
261 (Figure 4CD). These rows of elongated cells multiply, branching throughout the spur as
262 development proceeds.

263 *Average cell volume in whole spurs*

264 Based on the variation of the average cell volume during spur development, two phases can
265 be defined. From stage 01 to 06 (i.e. during spur formation), mean cell volume remains
266 relatively stable (on average between 550 and 900 μm^3) while cell number and total volume
267 of the spur increase, suggesting cell proliferation as the main process. From stage 07 to 10
268 (i.e. during spur growth), the mean cell volume increases continuously (until on average
269 5,000 μm^3 (stage 07) to 20,000 μm^3 (stage 10), in parallel with the total spur volume (Figure
270 5A). This observation is consistent with cell expansion as the main cellular process at work
271 during this second phase. In other words, between the two phases there is a 36-fold increase
272 in volume or a 3.3-fold increase in size. Note that the stage 09 seems particular, in which the
273 cell volume does not follow the overall trend.

274 At stage 06, the spur has six times more cells than in the previous stage (stage 06: 123,146
275 cells versus stage 05: 19,596 cells). At the following stage, there are 3 times less cells (Stage
276 07: 32,539 cells) (Figure 5B, Supplementary Fig. S2).

277 *Average cell volume in sectorised spurs*

278 Overall during development, an increase in cell volume is observed in the spur. However, at
279 each developmental stage, cell volume differs significantly among proximal, median and

280 distal sectors. The mean cell volume decreases and cell density increases towards the tip of
281 the spur. Cell density of the distal cells (sector D) is the highest and the cells are the smallest
282 (Stage 10 - Figure 6).

283 *Cellular anisotropy*

284 In the spur, cell orientation varies throughout development (Figure 7). They tend to be
285 orthogonal to the longitudinal axis at stages 01 and 10. At stages 02, 04, 05, and 06, cell
286 orientation follows the direction of the longitudinal axis. At stage 03 and stages 07 to 09,
287 cells are distributed in two intermingled main populations depending on their orientation. The
288 orientation of one cell population follows the longitudinal axis, whereas the orientation of the
289 other population is orthogonal to the longitudinal axis. These two populations are visible on
290 SEM images of tangential and longitudinal sections of spurs on mature petals or petals
291 collected at stages 07 (Supplementary Fig. S3).

292 *Curvature*

293 The virtual sections allowed us to study cell volumes and orientations across the spur (among
294 sectors and between the lower and upper sides). A difference between both sides appears at
295 stage 07, and a lower cell volume is observed in the upper side at stage 07 for all sectors. No
296 particular trend is detected among developmental stages and among sectors during
297 development (Supplementary Fig. S4).

298

299 **Discussion**

300 Studies on spur morphogenesis in angiosperms remain scarce and have mainly concerned
301 three unrelated genera, namely *Aquilegia* (Ranunculaceae), *Linaria* (Plantaginaceae) and
302 *Centranthus* (Caprifoliaceae). These studies are focused on the epidermal cells, or are based
303 on two-dimensional observations of this organ, often addressing the question of the origin of
304 the interspecific diversity within a genus (*e.g.* Puzey *et al.*, 2012; Galipot *et al.*, 2021;
305 Edwards *et al.*, 2022). Comprehensive 3-dimensional information on all cells of the spur (not
306 only the epidermal cells) is missing, hindering our full understanding of the mechanisms
307 involved in complex petal forms. The present study aimed at filling this gap, using a new
308 open source method of image processing developed for the purpose of this study. We observe
309 that the development of the spur of *Staphisagria picta* proceeds in two phases. The first phase
310 leads to the formation of an invagination by cell proliferation, whereas the second phase leads
311 to spur elongation by anisotropic cell expansion in two different directions. The final shape,
312 *i.e.* a thin and curved spur, results from the presence of cells oriented towards the spur cavity
313 from the start of elongation and, cells that remain smaller and more spherical than others
314 towards the distal part of the spur throughout development.

315 *Pattern of cellular processes during spur development*

316 Our results on the development of the spur in *Staphisagria picta* suggest a pattern marked by
317 an early phase of dominant cell proliferation that allows the formation of an invagination
318 which is deeper than wide and that may already be defined as a spur. Subsequently, a second
319 phase of directional (anisotropic) cell expansion dominates in most parts of the spur. These
320 results are similar to those obtained in *Aquilegia* species that have spurred petals, suggesting
321 convergence in cellular processes: a first phase of cell proliferation that stops early during

322 development, followed by a longer phase of anisotropic cell elongation. However, it is
323 difficult to go deeper in the comparison because the studies in *Aquilegia* focused on
324 epidermal cells (Puzey *et al.*, 2012) while we got data for the whole spur volume. In
325 particular, our spatial analysis of spur elongation along three sectors shows that this
326 elongation is not uniform within the spur. Cell expansion is concentrated in the proximal and
327 median parts of the spur, which have the largest width. The cells are smaller and more
328 spherical in the distal sector of the spur, corresponding to the tip. The second phase of
329 development is marked by cell expansion, present everywhere in the organ, except in the
330 most distal part. Moreover, cells have specific orientations throughout development. Cells
331 oriented along the longitudinal axis of the spur are present at all stages, except stages 01 and
332 10, suggesting cell elongation. A population of cells oriented orthogonally to the longitudinal
333 axis of the spur is also present, which may suggest thickening of the petal combined with
334 enlargement of the opening and internal cavity of the spur in its upper part.

335 *Curvature*

336 The detailed analysis of epidermal cells in *Aquilegia* petals bearing curved spurs (such as in
337 *A. canadensis*) revealed that there is a differential growth between the distal and proximal
338 surfaces of the spurs (relatively to the center of the flower), essentially due to differential cell
339 division (Edwards *et al.*, 2022). Our results do not support the hypothesis of a similar
340 differential growth of the curved spur of *Staphisagria* when all cells are considered.
341 However, they do reveal some complexity in the formation of the curvature since two cell
342 populations with orthogonal orientations (along the longitudinal axis of the spur as well as
343 orthogonal to this axis) are revealed at stages when curvature is achieved. This might be
344 explained by differential tissue formation and growth between the epidermis and the
345 parenchyma, suggesting different mechanisms in curvature formation in *Staphisagria* and
346 *Aquilegia*.

347 *Vasculature*

348 At stage 03, when the petal presents a flat blade and an emerging depression, three lines of
349 elongated cells oriented from the proximal part to the distal part of the petal are observed.
350 These elongated cells could be indicative of the vascular system. Petal vascularization was
351 studied in three species of Delphinieae (*Aconitum lasiocarpum*, *Delphinium elatum*, *D.*
352 *consolida*) (Novikoff and Jabbour, 2014), and in one species of Nigelleae (*Nigella*
353 *damascena*) (Deroin *et al.*, 2015), and the petals of all studied species have three vascular
354 bundles. These cells are visible in the raw confocal microscopy images. In the three-
355 dimensional reconstructions and in the volume distribution graphs, they are only visible in
356 stage 03. This is due to the increase in the number of parenchyma cells that makes the cells of
357 the vascular bundle undetectable. The observation of the confocal microscopy images shows
358 that these lines of elongated cells become more numerous and branched as the spur grows,
359 with the highest number of cells observed at stage 06. We hypothesize that this stage is
360 characterized by high cell proliferation, and formation and branching of the vascular system.
361 The vessels are tubular and made of lignified dead cells. This could explain why, at the
362 subsequent stages, the walls could no longer be detected by the software with, as a
363 consequence, a dramatic drop in the number of cells recognized.

364 *Diversity of spurs and short invaginations in Ranunculaceae and other eudicots*

365 Convergence in spur development between *Aquilegia* and *Staphisagria* appears through the
366 successive contribution of cell proliferation (mostly shape elaboration) and anisotropic cell

367 elongation (deepening). However, more detailed comparative analyses have still to be
368 conducted to determine the relative temporal contribution of the two phases to final shape in
369 different species. In American *Aquilegia species*, spurs present a large diversity in length
370 associated with the type of pollinators, and it has been shown that the duration of the phase of
371 anisotropic elongation largely accounts for such diversity (Puzey *et al.*, 2012). Actual spurs,
372 *i.e.* deeper than wide invaginations, evolved only twice in Ranunculaceae but other forms of
373 invaginations have been described in the family, in distantly related genera. This is the case in
374 *Aconitum* (Delphinieae), *Nigella* (Nigelleae), *Urophysa* (Isopyreae), *Actaea* (Cimicifugeae),
375 and *Coptis* (Coptideae) (reviewed in Delpeuch *et al.*, 2022). These invaginations are variable
376 in depth and width. Modeling petal shape based on a small set of morphogenetic parameters
377 could account for this diversity (Cheng *et al.*, 2023). The petals of *Nigella* species are
378 spurless, but their blade is deeply invaginated and present two lips. At the cellular level, the
379 development of the invagination of the petal of *Nigella damascena* is organized in two
380 successive steps of cell proliferation and cell expansion, as observed in *Staphisagria* and
381 *Aquilegia*. The first phase is responsible for most of the shape (Galipot *et al.*, 2021). The two
382 lips are formed by the differential expression domains of adaxial and abaxial genes during
383 morphogenesis (Yao *et al.*, 2019; Cheng *et al.*, 2023). Adjusting the values of the
384 morphogenetic parameters makes it possible to create the various petal shapes found in the
385 genus (Cheng *et al.*, 2023). How and if the morphogenesis of the invagination could relate to
386 spur formation in the sister group Delphinieae remains to be explored in detail.

387 In the non-Ranunculaceae species in which spur development was studied, the relative
388 contribution of each type of cellular processes varies. In *Centranthus ruber*, spur growth
389 initially involves diffuse cell divisions, and then, from 30% of its final length until anthesis,
390 cell elongation is mainly responsible for spur extension. Thus, it is a period of anisotropic cell
391 elongation, with uniform elongation along the longitudinal axis of the spur, that primarily
392 contributes to the length of the mature spur (Mack and Davis, 2015). In *Linaria*
393 (Plantaginaceae), although both processes are present, cell proliferation was shown to be the
394 primary mechanism involved in spur growth, and responsible for the difference in spur length
395 among different species (Cullen *et al.*, 2018).

396 Genetic origin of spurs has been investigated in a handful of species in eudicots and suggest
397 that different genes are implicated. In *Aquilegia*, transcription factors of different families
398 (TCP, ARF, C2H2 Zinc Finger) play a role in spur formation, some of which involved in
399 auxin signaling (Yant *et al.*, 2015; Ballerini *et al.*, 2020; Zhang *et al.*, 2020). In Delphinieae,
400 the presence of a petal spur is closely linked to bilateral symmetry. A VIGS gene inactivation
401 study in *Delphinium ajacis* has revealed interplay between a paralog of the petal identity gene
402 *APETALA3-3* and a paralog of *CYCLOIDEA* (*CYL2b*) in the dorsal identity, including petal
403 spur formation (Zhao *et al.*, 2023). Transcription factor genes of the *KNOX* family have been
404 suspected to direct spur formation in *Linaria* (Box *et al.*, 2011).

405 *Methodological perspectives*

406 Automated analysis now makes it possible to study organ morphogenesis in 3D, across all
407 cell layers. This method of analysis allows the inclusion of large sample sizes and is therefore
408 well suited to the study of developmental sequences. Producing confocal microscopy images
409 transverse to the longitudinal axis of the spur would allow a more detailed study of the
410 cellular mechanisms involved in the enlargement of the spur opening and cavity, and would
411 also provide information on the formation of the curvature. It would also be interesting to
412 separate the epidermal cells from the parenchymal cells to compare cellular processes
413 between these tissues, and also to compare the results with those obtained from the study of

414 the epidermis. The automation of the analyses allows now to carry out, in a more user-
415 friendly way, 3D studies of organ morphogenesis on all cell layers in the framework of a
416 developmental sequence, and including a larger number of samples.

417

418 **Conclusion**

419 The way in which similar morphological structures are obtained through evolutionary
420 convergence is a fascinating question. Here, we address this question by focusing on floral
421 spurs. These structures are tightly associated with reproduction, by being invaginations in
422 which nectar accumulates, providing a resource for potential pollinators. The analysis of the
423 spur of *Staphisagria picta* revealed a process marked by an early phase of dominant cell
424 proliferation, followed by a phase of anisotropic (directional) cell expansion, revealing that
425 the convergence in form between the spurs of *Staphisagria* and *Aquilegia* is obtained by
426 partially similar development processes. The method developed in the present study allowed
427 precise 3D comparative studies at the cellular level. The recognition, identification, and
428 extraction of information can be performed for each cell in a sample of organs of various
429 sizes, typically belonging to a developmental sequence, using the same segmentation method.
430 This allowed us to obtain a large amount of data and to perform a comprehensive 3-
431 dimensional analysis of the morphogenesis of a complex structure. The same methodology is
432 applicable to other complex organs and structures.

433

434 **Supplementary data**

435 Supplementary Figure S1: Developmental benchmark for developmental stages of
436 *Staphisagria picta* flowers.

437 Supplementary Figure S2: Visualization of vascular tissue and parenchyma in images
438 generated by confocal microscopy.

439

440 Supplementary Figure S3: Visualization of epidermal cells, cells on longitudinal and
441 tangential sections, and vascular tissue generated by scanning electron microscopy.

442 Supplementary Figure S4: Study of the curvature of the spur.

443

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446 providing seeds of *Staphisagria picta*, and to the Jardin Botanique de Launay (Orsay, France)
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450

451 **Author contributions**

452 PD, SN and FJ designed the study. PD and KB performed the developmental analysis. PD
453 and AP designed the pipeline for 3D image processing. PD wrote the first draft of the
454 manuscript, SN, FJ and CD contributed to the writing. All authors contributed to the article
455 and approved the submitted version.

456

457 **Conflicts of interest**

458 No conflict of interest declared.

459

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462 Evolution) and UMR 7205 - ISYEB (Institut de Systématique, Evolution, Biodiversité).

463

464 **Data availability**

465 The data underlying this article are available in the article and in its online supplementary
466 material.

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Figure legends

Figure 1: Spur diversity in angiosperms (a) *Orchis militaris*, (b) *Anacamptis pyramidalis*, (c) *Viola riviniana*, (d) *Tropaeolum tuberosum*, (e) *Valeriana erotica*, (f) *Linaria vulgaris*, (g) *Aquilegia vulgaris*, (h) *Delphinium ajacis*. Photographs: Sophie Nadot except (g): Wikimedia © Aiwok.

Figure 2: Floral morphology of *Staphisagria picta* and simplified phylogeny of Ranunculaceae, with tribes characterised by spurred flowers highlighted Ranunculaceae tribes. A: Structure of the flower of *Staphisagria picta*: (1) flat petals, (2) spurred petals, (3) sepals. B: Phylogenetic relationships among Ranunculaceae tribes (based on Zhai *et al.*, 2019); tribes including taxa characterized by flowers with spurred petals are highlighted in yellow (Delphinieae) and blue (Isopyreae). On the right hand of the figure: flowers of *Staphisagria picta* (top) and *Aquilegia vulgaris* (bottom) and details of their petals. The orange dot indicates the insertion point of the organ on the floral receptacle. The corolla of *S. picta* consists of two flat lateral petals and two spurred dorsal petals. The pair of dorsal petals is nested within the spurred sepal.

Figure 3: Orientation of petals in space and along the developmental sequence. (A) Definition of sectors: proximal, median, distal and lower and upper sides, (B) Definition of the spur opening and internal cavity, (C) photographs of petals along the developmental sequence. The orange dots indicate the petal insertion point on the floral receptacle. Scale: stages 01–05: 150 μ m, 06: 1mm, 07–10: 2mm).

Figure 4: Different steps in the visualization of results illustrated by adaxial views of stage 03 petals. A: confocal microscopy images, only one cell layer is shown but it is possible to move layer by layer within the petal. B: different cell shapes: (1) small parenchyma cells, (2) large parenchyma cells, (3) regular, large epidermal cells present in a single cell row, (4) elongated cells belonging to the vascular system. C: visualization of the results by reconstruction of the petals in 3D with *in silico* staining of the cells according to their volume. The three lines corresponding to the vascular system are indicated by white arrows. D: histogram of cell volume distribution. The orange ovals indicate the petal insertion point on the floral receptacle.

Figure 5: Variation of spur and cell volume during development. Left, description of the petal shape, and evolution of mean cell volume compared to whole spur volume during development. All values are in logarithmic scale. Scale: stages 01–05: 300 μm , 06: 2 mm, 07–10: 4 mm). The yellow dots represent the area of insertion to the floral receptacle. Right, boxplots represent the volume of cells at each stage. Blue and yellow areas represent the first and second phase of development, respectively. N: cell number per stage.

Figure 6: Variation of cell volume and density during spur development. Left, boxplots representing cell volume (μm^3) according to stages and sectors. Right, representation of cell density on the proximal (P), median (M) and distal sector (D) along the developmental sequence. All values are in logarithmic scale.

Figure 7: Quantification of cellular anisotropy during spur development. Plots representing the deviation angle of the directional axis of cells from the direction of the longitudinal axis of the petal for the spur. The black curve represents the distribution of randomly spheric oriented cells. Significant orientation (anisotropy) appears as peaks above this black curve. The color code refers to the developmental stages defined in the previous figures.

Supplementary data legends

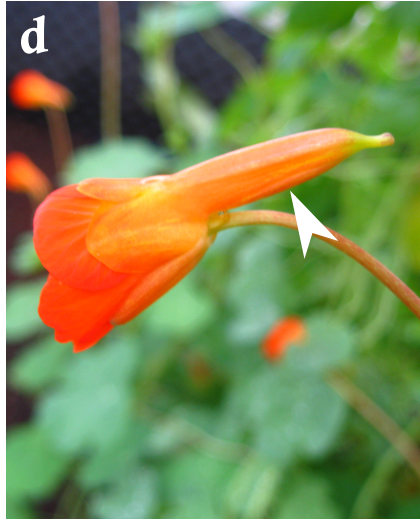
Supplementary Figure S1: Developmental benchmark for developmental stages of *Staphisagria picta* flowers. Figure adapted from (Delpeuch *et al.*, 2022). Blue, yellow and red colours refer to stamen, carpel, and petal development, respectively. The numbers in the purple disks correspond to the developmental stages targeted in the present study.

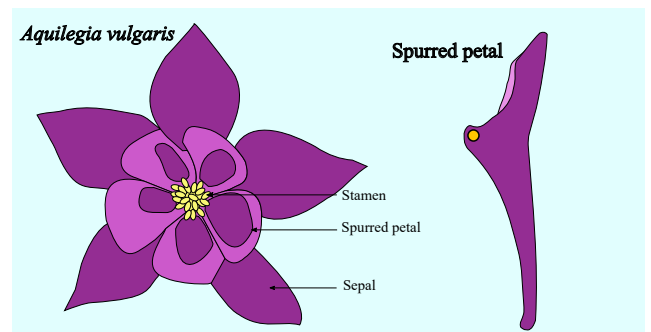
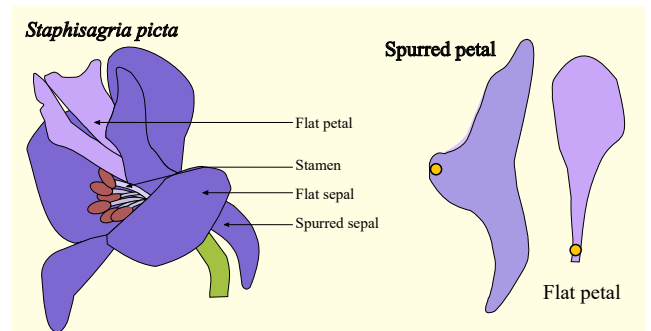
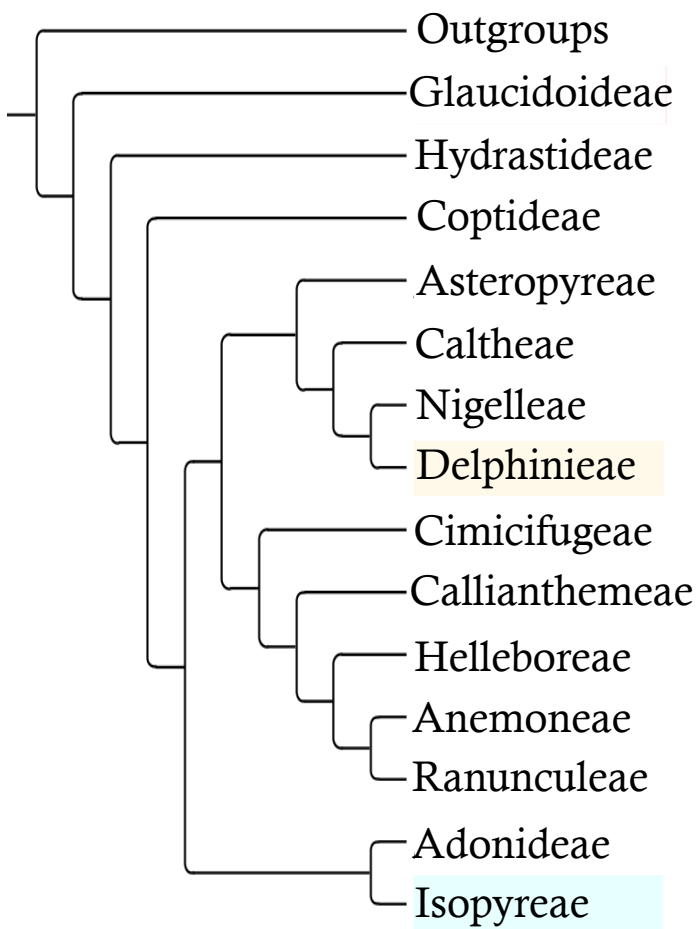
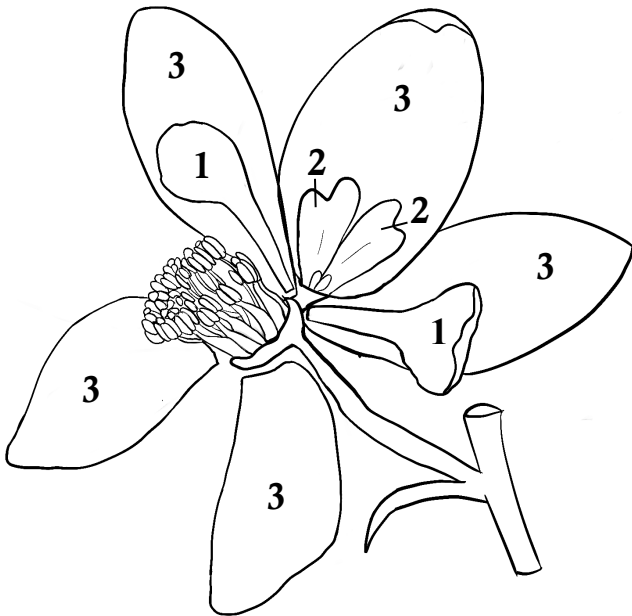
Supplementary Figure S2: Visualization of vascular tissue and parenchyma in images generated by confocal microscopy at stages 03 (top) and 09 (bottom). Yellow and blue arrows indicate respectively vascular tissue and parenchyma cells.

Supplementary Figure S3: Visualization of epidermal cells, cells on longitudinal and tangential sections, and vascular tissue generated by scanning electron microscopy at stage 07 and on mature petals. Blue and yellow arrows indicate respectively elongated cells and cells potentially oriented perpendicularly to the cutting plane.

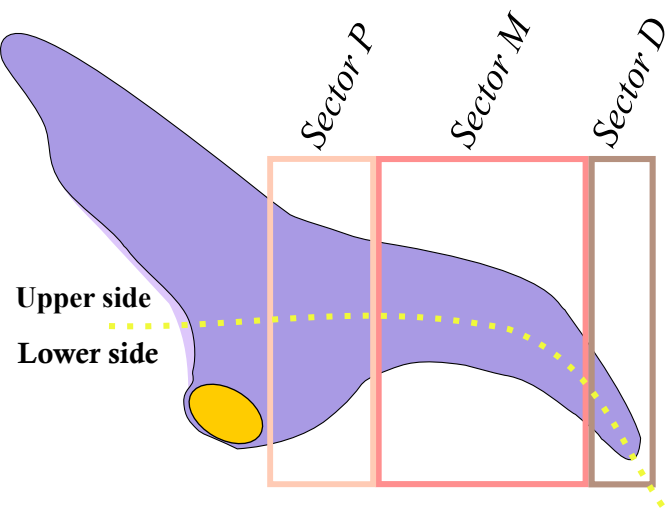
Supplementary Figure S4: Study of the curvature of the spur. (A) Variations of cell volumes according to sectors and faces. The black spot in the boxplot is the average. (B) Variations of cell orientations. Plots represent the deviation angle of the directional axis of cells from the direction of the longitudinal axis of the petal for the spur. The small grid, on top left, indicates the position of the measurement. The color code refers to the developmental stages

defined in the previous figures. The orange dot indicates the insertion point of the organ on the floral receptacle.

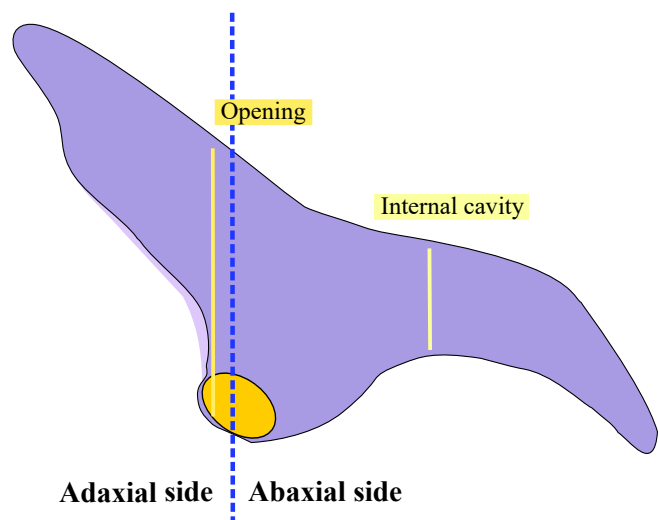




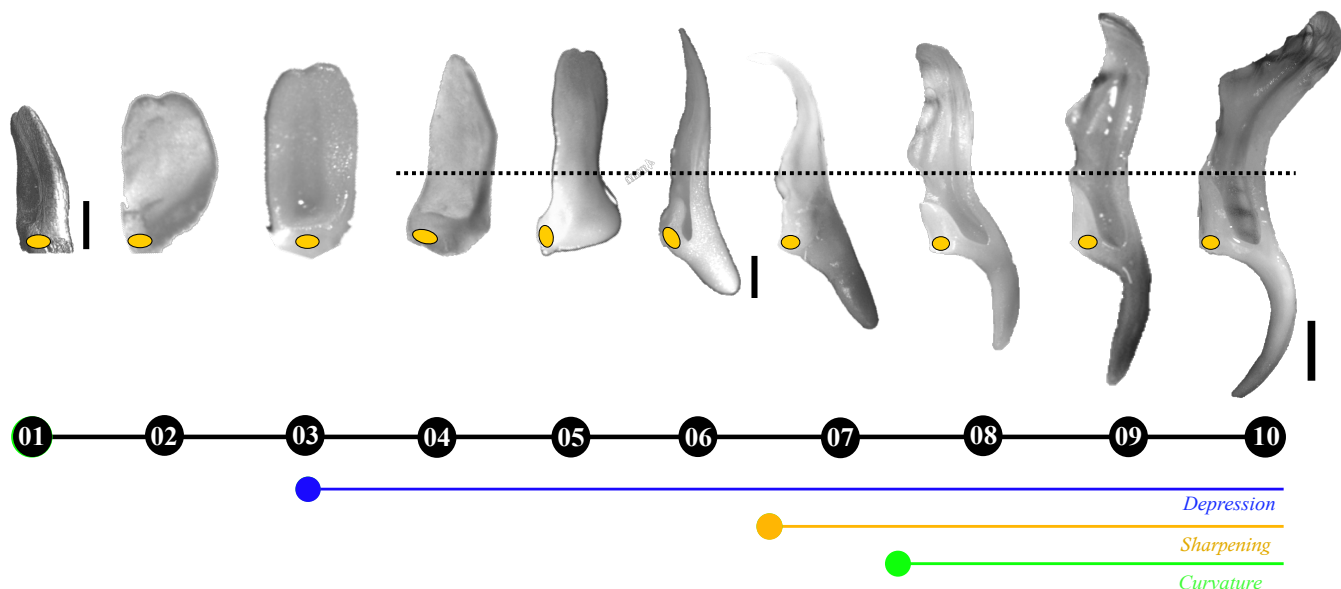
A



B

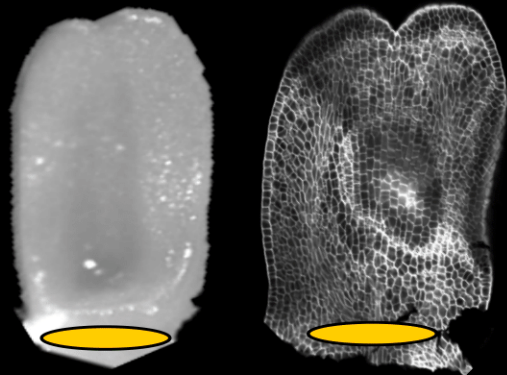


C

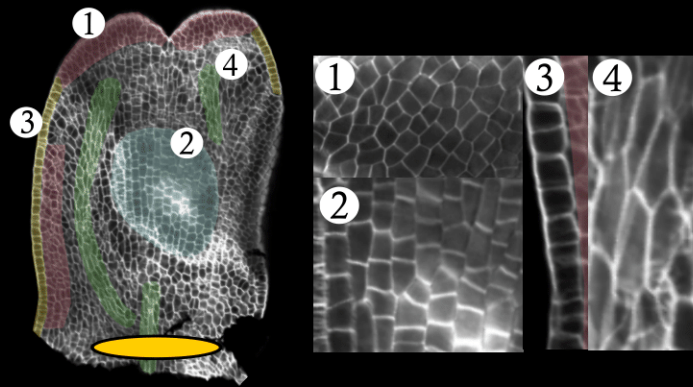


A- Image acquisition

Dissected petal Confocal microscopy image



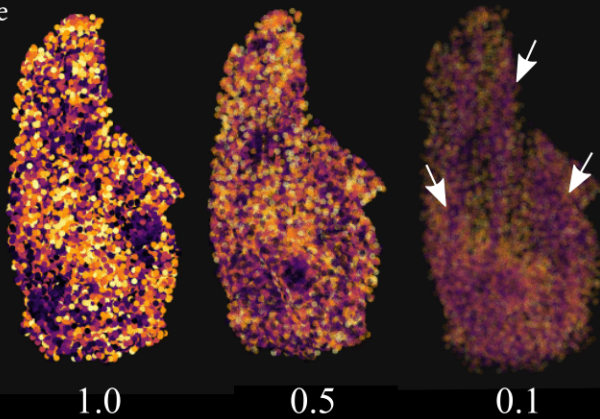
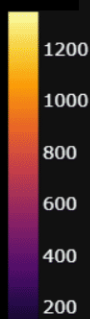
B- Cellular diversity on petals



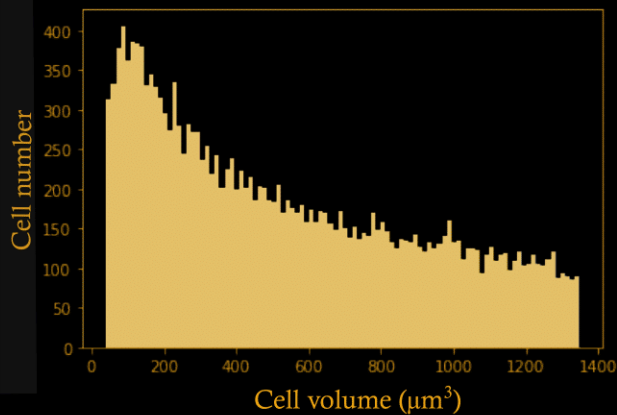
C- 3D visualization

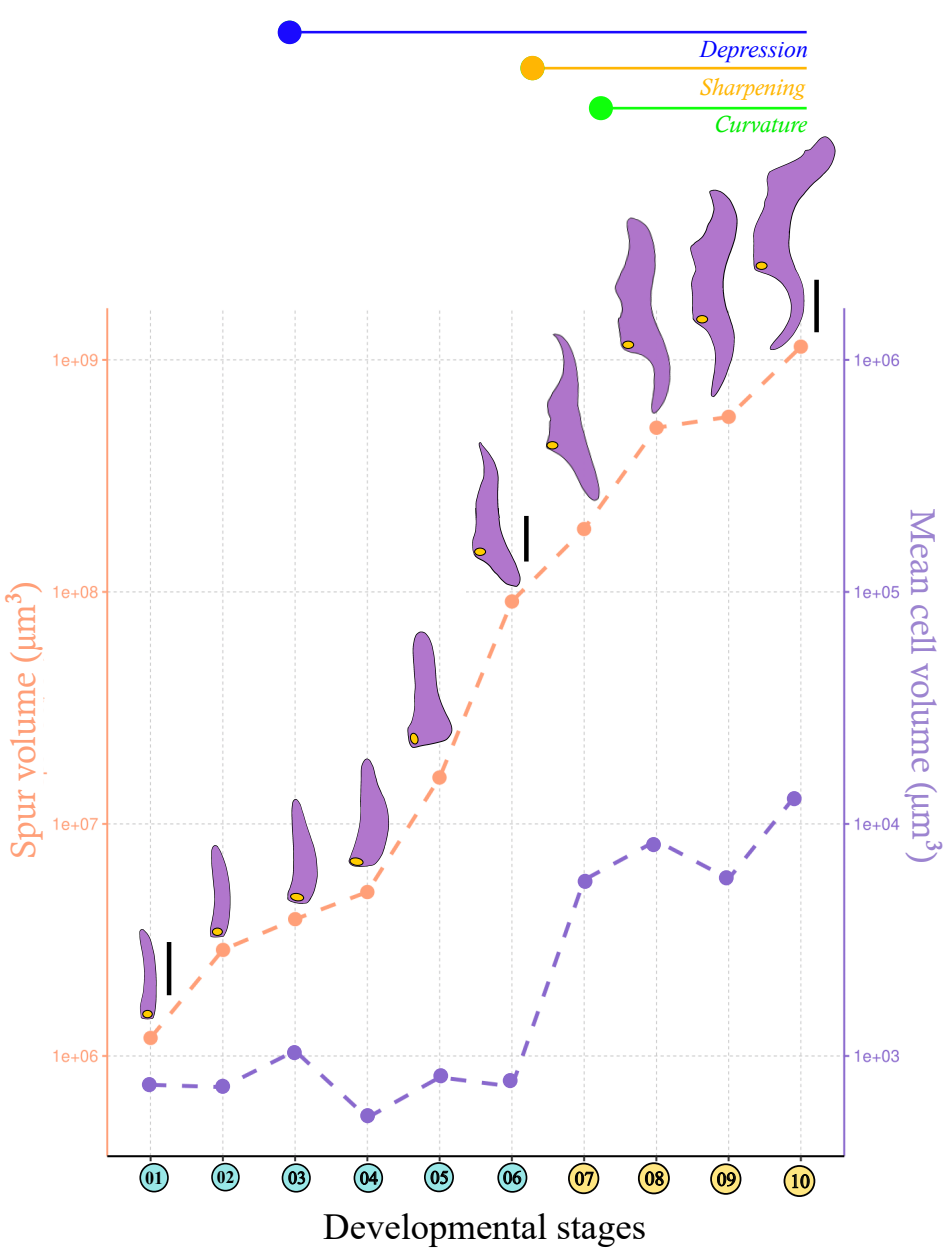
- Different dots opacities to see all cell layers -

Cell volume
(μm^3)

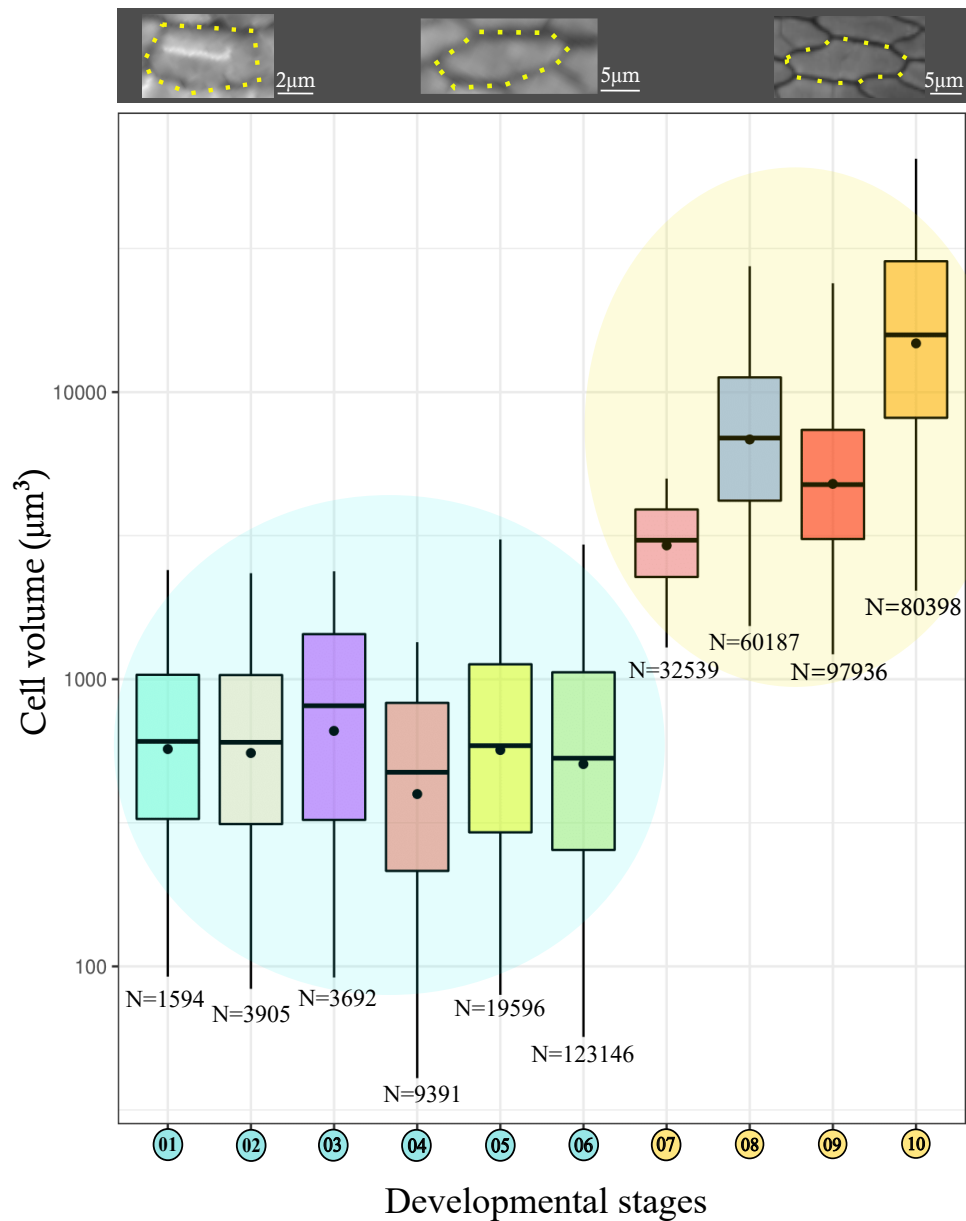


B- Volume distributions on stage 03

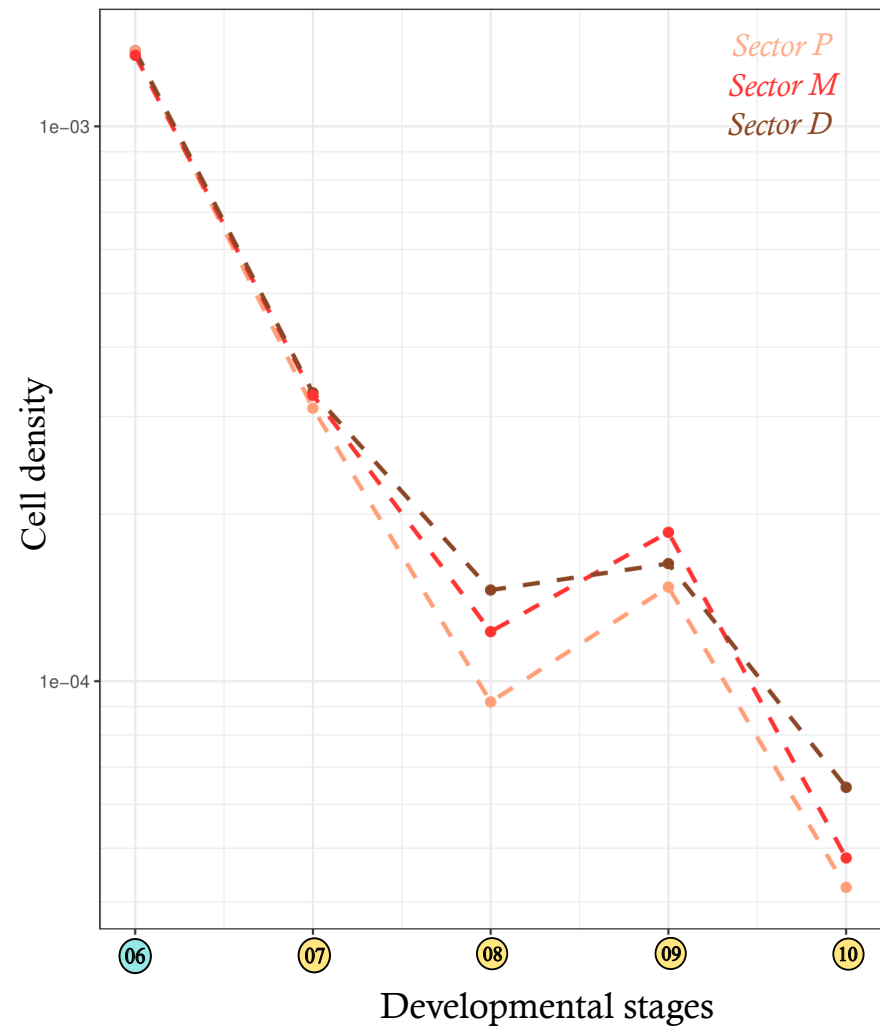
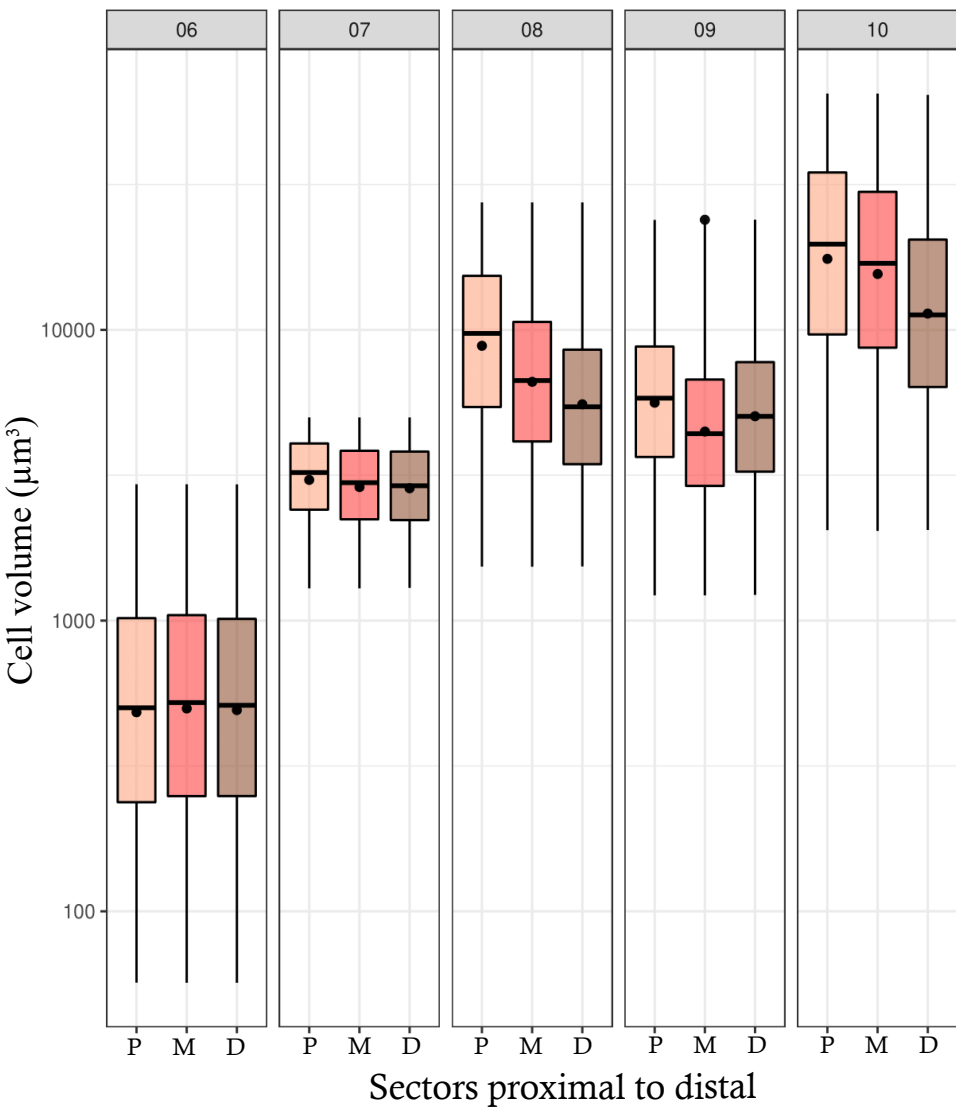




Cell pictures in confocal microscopy



Developmental stages



Developmental stages

