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Apomixis beyond trees in the Brazilian savanna: new insights from the orchid Zygopetalum mackayi

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Abstract

Knowledge on the biology of apomictic tropical plants is still fragmentary. Studies on trees from the Brazilian savanna show apomixis is mainly sporophytic, facultative and associated with polyploidy and polyembryony. Here we show, for the first time, how reproduction mode is associated with chromosome numbers and polyembryony in an herbaceous plant from the Brazilian savanna, the orchid *Zygopetalum mackayi*. We described megasporogenesis and megagametogenesis in all three cytotypes of this species and clarify apomixis is strictly sporophytic, facultative and restricted to triploids and tetrapoloids, while seed formation is strictly sexual in diploids. Polyembryony is mainly a result of apomixis, but also a consequence of the occurrence of multiple archesporium in all cytotypes. Fruit set is higher in tetraploids compared to other cytotypes and suggest the occurrence of a late-acting self-incompatibility system in diploids. Contrary to other Brazilian savanna species, apomixis in *Z. mackayi* does not allow for reproductive assurance. We hypothesize range expansion of tetraploids as a consequence of higher fruit set compared to diploids and polyembryony associated with putative physiological plasticity increased by polyploidy. Biological consequences of apomixis may be more diverse in tropical biomes than previously described for temperate species.

Key Message

Sporophytic apomixis in *Zygopetalum mackayi* orchids from the Brazilian savanna is associated with polyploidy, polyembryony and high self-compatibility. Wide geographical range of this species is associated to polyploidy, not apomixis.

Introduction

Elucidate the emergence, maintenance, and diversification of asexual lineages is one of the most relevant debates of evolutionary biology (Hojsgaard et al. 2014). Apomixis, the production of seeds asexually, is known for more than 400 species, 293 genera, and 78 phylogenetically diverse families of angiosperms (Hojsgaard et al. 2014; Barcaccia et al. 2020). Apomictic embryos may originate from three different developmental mechanisms (Koltunow 1993). In sporophytic apomixis (adventitious embryony), adventitious embryos originate directly from somatic cells within the ovule. By contrast, in gametophytic apomixis the embryo develops via egg cell parthenogenesis in an unreduced embryo sac, originated from a somatic cell, which bypasses regular meiosis. This unreduced embryo sac may originate from a megaspore mother cell (diplospory) or from a nucellar cell (apospory). Polyembryonic seeds with sexual and asexual embryos may be associated with both sporophytic and aposporous apomixis (Naumova 1993).

Most knowledge on the origin and diversification of apomictic plant lineages is based on gametophytic apomictics from northern latitudes (Hojsgaard and Hörandl 2019). Compared to their sexual relatives, northern hemisphere apomictic plants usually are polyploids, have larger distributions, occur at higher altitudes and colonize previously glaciated areas (Hojsgaard et al. 2014). For tropical species, the

association of apomixis and biogeographic traits is still poorly known. Available studies show apomixis in neotropical plants is mainly sporophytic, facultative and associated with polyploidy and polyembryony (e.g. Mendes-Rodrigues and Oliveira 2012; Alves et al. 2016). However, knowledge on the biology of apomictic tropical plants is still fragmentary and mainly concentrated on trees and shrubs from the Brazilian savanna. In this scenario, apomictic herbaceous plants emerge as a potential new model for the study of apomixis in the Neotropics.

Orchids comprise one of the most diverse lineages of angiosperms. Out of 905 accepted orchid genera (WFO 2022), apomictic species are known for 22 genera and less than 1% of species, which are mainly sporophytic (Hojsgaard et al. 2014; Zhang and Gao 2018; Xiao et al. 2021). The species *Zygopetalum mackayi* Hook. is the only orchid to combine apospory and sporophytic apomixis (Hojsgaard et al. 2014). Almost a century ago, apomictic reproduction was described for this species, a leaf-litter orchid which occurs in high-elevation rocky complexes within the Brazilian savanna and Atlantic forest (Gomes et al. 2018). Suessenguth (1923) described the simultaneous occurrence of gametophytic and sporophytic apomixis, the production of polyembryonic seeds, and the occurrence of polyploids in this species. He also suggested no sexual embryos are formed, and that the pollen tube was necessary only to stimulate the development of apomictic embryos. Later, Afzelius (1959) also proposes the occurrence of obligate apomixis in *Z. mackayi* but reported embryo formation strictly by sporophytic apomixis. However, both studies were based on one or two specimens and only performed self-pollinations or cross-pollinations with phylogenetically very distinct species (*Calanthe* and *Coelogyne*). Later, Gomes et al. (2018) showed *Z. mackayi* exhibits three different cytotypes (diploid, 2N = 48; triploid, 2N = 72; and tetraploid, 2N = 96). Diploids and tetraploids are geographically structured and associated with different climatic conditions, while triploids are F1 hybrids that occur in a contact zone where diploids and tetraploids meet (Gomes et al. 2018; Moura et al. 2020). In addition, studies of reproductive biology (Campacci et al. 2017) and population genetics (Moura et al., 2020) suggested *Z. mackayi* reproduces mostly sexually and facultatively by apomixis, being the embryo formation pollination-dependent for fruit development (Campacci et al. 2017).

Given recent studies on cytogenetics, genetics, reproductive biology, and ecological niche modelling of *Z. mackayi* (Campacci et al. 2017; Gomes et al. 2018; Moura et al. 2020) and the inconsistency of previous studies on the developmental mechanism of apomixis (Suessenguth 1923; Afzelius 1959), we here clarify the relationship among apomixis, chromosome numbers and polyembryony in this species. Specifically, we propose to answer the following questions: (1) Is apomixis facultative and expressed together with sexual reproduction? (2) What is the origin of apomictic embryos? (3) Is apomixis associated with polyploidy? (4) Is polyembryony associated with apomixis? Results are discussed in the light the role of apomixis in plant diversification in the Brazilian savanna.

MATERIAL AND METHODS

Zygopetalum mackayi flowers mainly in the dry season, between April and July, with a second less intense peak between December and January (Campacci et al. 2017; Nunes et al. 2017). Plants were

cultivated from January 2016 to October 2017 in the orchid nursery of Universidade Estadual de Campinas (São Paulo, Brazil). A total of 53 specimens were used in this study. Vouchers were deposited at UEC (Table 1).

To define embryo origins and describe megasporogenesis and megagametogenesis, we performed manual self-pollinations in first day flowers considering 24 flowers of diploids individuals (*n* = 17 specimens), 17 flowers of triploids individuals (*n* = 6), and 79 flowers of tetraploids individuals (*n* = 30) (Table 1). Flowers and fruits in different stages of development were collected (1-100 days after pollination; DAP) (Table 1). We described ovule development and morphology considering sexual and apomictic processes. For that, we fixed the samples by immersion in a solution of 4% formaldehyde, 2.5% glutaraldehyde in 0.05 M phosphate sodium buffer (modified from Karnovsky 1965), then gradually dehydrated in an ethanol series before being embedded in resin (Leica Historesin®). Sections of 3–5 µm thick were obtained with a manual rotary microtome (Leica®), stained with 0.05% Toluidine Blue 0.05% in citrate buffer, pH 4.5 (Sakai 1973) and mounted with synthetic resin Entellan (Merck®). The analyses were performed using an Olympus BX51 optical microscope equipped with a digital camera Olympus DP71.

As self-pollination treatments resulted in high fruit abortion, especially in diploids, we also analyzed pollen tube development in aborted flowers and fruits to search for putative anomalies. For that, we collected the distal portion of the gynostemiumm and/or the medium portion of fruits at early abortion. Samples were fixed in ethanol 70% for 48 h later soften in a NaOH 10N solution at 60°C for 15 min and then washed in distilled water and kept overnight in a 1% anilin blue solution in potassium phosphate buffer, pH 7.0 (modified from Martin 1959). Analyses and photomicrographs were made using a light microscope Olympus® DP71 equipped with epifluorescence.

RESULTS

Sexual development of ovules and seeds

All cytotypes showed similar patterns of ovule and seed development. The ovary has three carpels divided into six valves, three fertile, with the presence of the placental region, and three sterile (Fig. 1A). In the anthetic flower, the fertile valves present only primordia ovules (Fig. 1B). After manual self-pollination, the pollen grains germinate and the pollen tubes grow along the stylar canal and, concomitantly, the placenta proliferates through intense mitotic activity (Fig. 1C). The pollen tubes reach the base of the fruit about 13 DAP and stay in the placental region until the ovule matures (Fig. 1C). The ovules start to develop with approximately 20 DAP (Table 1). The ovule differentiation is not synchronous within the same fruit nor among cytotypes (Table 1).

In each ovule primordium, a cell from the subepidermal layer differentiates into an archesporial cell, with an evident nucleus (Fig. 1D). In this phase of development, periclinal cell divisions occur in the nucellar epidermis, leading to the formation of the outer and inner integuments (Fig. 1D-E). The archesporial cell increases in volume and does not divide, directly originating from the megaspore mother cell (MC;

Fig. 1E-F). The first phase of the meiotic division of the MC results in the formation of a megaspore dyad (Fig. 1G-H) and the second phase of the meiotic division originates a tetrad of megaspores (Fig. 1I-J). At this stage, the inner integument has elongated, and its margins completely cover the nucellar epidermis, delimiting the micropyle (Fig. 1G-J). The calazal megaspore becomes functional and the three micropylar megaspores degenerate (Fig. 1K).

The functional megaspore increases its volume, and the first mitotic cycle occurs producing a binucleate megagametophyte. Subsequently, a large central vacuole is formed, and each nucleus moves into one pole of the megagametophyte (Fig. 1L). The two nuclei simultaneously undergo the second mitotic cycle, originating a tetranucleate megagametophyte (Fig. 1M), followed by the final third mitotic cycle, forming an octanucleate megagametophyte. During cellularization, one nucleus of the chalazal pole and one of the micropylar pole migrate to the center of the megagametophyte, constituting the polar nuclei of the central cell (Fig. 1N). The remaining nuclei of the micropylar pole organize into two synergids and an egg cell in a triangular arrangement (Fig. 1O), and the three remaining nuclei of the chalazal pole form the antipodals (Fig. 1N). These patterns indicate a *Polygonum*-type of embryo sac development (monosporic, eight-nucleate and seven-celled). The ovules are anatropous, tenuinucellate and bitegmic at this stage.

Fertilization occurs 42–64 DAP and is identified by dense cytoplasmic staining of the synergid. Zygote organization occurs 44–66 DAP (Fig. 2A-C; Table 1). The polar nuclei fuses to the male gamete nucleus forming the primary endosperm nucleus, which divides to form up to four nuclei (Fig. 2C). The first mitotic division of the zygote is asymmetric and generates a smaller apical cell and a larger basal cell (Fig. 2D). The apical cell forms the embryo, and the basal cell originates the suspensor (Fig. 2E-G). The apical cell undergoes more divisions without a defined pattern, originating the pro-embryo. The suspensor cells become elongated and vacuolized, occupying a large portion of the seed (Fig. 2E- G). During the embryogenesis, the cells of the inner integument degenerate, while the outer integument elongate to form the seed coat (testa) (Figs. 2G-I). As the embryo grows, the suspensor becomes compressed and degenerates. The mature seed shows a transparent testa, formed by a single layer of lining cells, which protects the globular embryo (Fig. 2I). In mature seeds, there is no differentiation of meristems and cotyledons in the embryo (Fig. 2I).

We rarely observed the differentiation of two MCs in the same ovule in all three cytotypes (multiple arquesporium) (Figs. 3A-C). When this phenomenon occurred, the MCs underwent meiosis and formed two tetrads of megaspores, in which the chalazal megaspores are functional (Figs. 3A-C). The megaspores underwent three mitotic cycles followed by cellularization, originating two reduced megagametophytes within the same ovule (Fig. 3C).

Sporophytic apomixis originating supernumerary embryos in polyploids

In triploid and tetraploid cytotypes we observed the differentiation of nucellar cells in the micropylar region of the megagametophyte (Fig. 3D-G). These cells increased in volume and invaded the interior of the megagametophyte and were identified as adventitious embryos precursor cells (AEPs). We observed

the differentiation of up to three AEPs in the same ovule (Fig. 3H). AEPs can be differentiated from the egg cell by its peripheral and lateral position in the megagametophyte and by its dense cytoplasm, centralized nucleus and thickened cell wall. AEPs remained unchanged until pollen tube penetration into the synergid and the double fertilization occurred (Fig. 3F-I). Their development occurred concomitantly with the sexual embryo or after the initiation of the sexual embryo development (Fig. 3J-L). Further distinction between adventitious and sexual embryos from this stage was difficult, since they were structurally similar, and the adventitious embryos were in the micropylar region (Fig. 3K; 3M-O). However, while the sexual embryo suspensor was located in the micropylar region of the seed (Fig. 3M), the suspensor of the adventitious embryo was observed in both the micropylar and callazal regions (Figs. 3M-O).

Pollen tube development

Fruit set from self-pollinations in diploids resulted in higher abortion rates (66.2%), followed by triploids (57.1%) and tetraploids (33.3%). We did not observe in aborted flowers any interruption in pollen tube growth neither irregular callose deposition in any cytotype (Fig. 4A-K). In all cytotypes, pollen grains germinated in the stigma and pollen tube developed into the style 3–4 DAP (Fig. 4A; D). After nine DAP, pollen tubes covered almost all the style (Fig. 4B, E), and at 13 DAP, pollen tubes reached the gynostemium base and entered the fruit locule (Fig. 4C; F). Pollen tubes reached the fruit locule in aborted fruits of diploids (Fig. 4A-C) and tetraploids (Fig. 4J-K), but we have no data for triploids. Moreover, we did not observe placental differentiation and ovule formation in diploid aborted fruits regarded DAP period (Fig. 4G-I). Placenta and ovule primordia initiate development in tetraploid aborted fruits, but they cease and degenerate (Fig. 4J).

DISCUSSION

In this study we confirmed the occurrence of sporophytic apomixis of *Z. mackayi* as we were able to identify differentiation of nucellar cells into AEPs in triploids and tetraploids. Contrary to what Suessenguth (1923) and Afzelius (1959) pointed out, megasporogenesis and megagametogenesis occur regularly in all cytotypes of *Z. mackayi*, which excludes the occurrence of gametophytic and/or obligate apomixis in this species. Apomixis in *Z. mackayi* is, therefore, facultative and sporophytic, and associated with the polyploid cytotypes. Exclusive sexual reproduction occurs only in diploid individuals of *Z. mackayi*. Sexuality in all cytotypes has been proven by pollen tubes remains in the micropylar region of penetrated synergids, zygote formation, and polar nuclei fertilization, indicators of sexual events.

Apomixis in *Z. mackayi* is also dependent on pollination as pollen tube growth triggers ovule development in all cytotypes, with the development of apomictic embryos beginning after the sexual one, but before sexual fertilization. This contrasts with most other apomictic angiosperms (Johri 1992), in which pollination followed by fertilization of polar nuclei and endosperm formation is required for apomictic embryo development (pseudogamy *sensu* Nogler 1984). Contrary to other angiosperms, the endosperm in orchid seeds is non-functional, as it is never formed or degenerates after polar nuclei

fertilization (Yeung 2017). Therefore, male sexual function must be conserved in orchids not for endosperm formation, but because pollination is necessary for ovule development (Mayer et al. 2021). Dependence on pollination for ovule development may be an additional, yet unexplored, constraint determining the lack of more autonomous apomixis in orchids, a rare phenomenon in the family, known for *Cynorkis* spp. (Veyret 1972); *Genoplesium apostasioides* (Fitzg.) D.L.Jones & M.A.Clem. (Sorensen et al. 2009), *Habenaria malintana* (Blanco) Merr. (Zhang and Gao 2018), *Rhomboda tokioi* (Fukuy.) Ormerod (Xiao et al. 2021), *Spiranthes cernua* (L.) Rich. (Catling 1982; Schmidt and Antlfinger 1992), *Zeuxine strateumatica* (L.) Schltr. (Seshagirah 1941).

We also showed polyembyony in *Z. mackayi* is caused by two different processes. Besides the production of adventitious embryos from nucellar cells of triploids and tetraploids, polyembryonic seeds may also result from multiple archesporium in all cytotypes. The occurrence of multiple archesporium is a pre-meiotic event, that originates two identical megaspore mother cells that will develop twin tetrads, followed by two independent embryo sacs within a single ovule. This is a rare event in the Orchidaceae, so far reported only for *Lecanorchis japonica* Blume (Johri et al. 1992). Although the production of adventitious embryos is much more common than the occurrence of multiple archesporial cells, polyembryony should not be used as direct evidence of apomixis in this species. Also, it is possible that monoembryonic seeds bear a single apomictic, embryo instead of a sexual one, as it is not possible to distinguish between them when fully matured.

In orchids, apomixis is rare, phylogenetic and geographically spread and associated with polyploidy and terrestrial habit (Zhang and Gao 2018; Huang et al. 2009; Xiao et al. 2021; Supplementary Table S1). Besides *Z. mackayi*, there are eight confirmed apomictic orchid taxa exhibit sporophytic apomixis (Supplementary Table S1). Of these, *Genoplesium apostasioides* (Fitzg.) (as *Corunastylis apostasioides*) is the only to also reproduce by gametophytic diplosporic apomixis (Sorensen et al. 2009). Exclusive gametophytic apomixis is confirmed only for diplosporic *Rhomboda tokioi* (Fukuy.) Ormerod (Xiao et al. 2021). Like *Z. mackayi*, other facultative apomictic orchids also correspond to polyploid complexes in which species boundaries are blurred (e.g. *Spiranthes cernua*, Pace and Cameron 2017; *Nigritella nigra*, Hédren et al. 2000). Facultative apomixis is known for terrestrial orchids only and it is geographically widespread (Catling 1982; 1987; Teppner 1996; Huang 2009). A putative exception is *Epidendrum nocturnum*, which has been described as a facultative sporophytic apomictic species but grows as epiphytes or rupicolous in the neotropics (Veyret, 1982; Stort and Pavanelli, 1985). However, we did not consider this species in our survey as we found evidence of apomixis uncertain. Although apomixis is rare in the Orchidaceae, it has been recognized as a strategy to circumvent pollination limitation (Neiland and Wilcock 1998). More studies on orchid reproductive biology are needed, especially concerning tropical and epiphytic species, to understand the real breadth of apomixis in this family.

Apomixis role in the Brazilian savanna

Ecological consequences of apomixis in *Z. mackayi* differs from other apomictic plants from the Brazilian savanna. Previous studies with tree species report facultative sporophytic apomixis associated

with polyploidy, polyembryony and pseudogamy in *Eriotheca* spp. (Malvaceae), *Handroanthus* spp., *Anemopaegma* spp. (Bignoniaceae) and *Inga laurina* (Sw.) Willd. (Fabaceae) (Sampaio et al. 2013; Alves et al. 2016; Mendes et al. 2018; Mendes-Rodrigues et al. 2019). Species of tribe Miconieae (Melastomataceae) from the Brazilian savanna have a more diverse sort of apomictic mechanisms besides the ones reported to other families, including autonomous endosperm formation and apospory (Goldenberg and Shepard 1998; Mendes-Rodrigues and Oliveira 2012; Caetano et al. 2018). Contrary to *Z. mackayi*, apomixis in all these plants allows for reproductive assurance, as autonomous self-pollination occurs or it is not needed. Reproductive assurance coupled with larger geographical ranges of apomictics in the Brazilian savanna was first suggested by Mendes-Rodrigues et al. (2019) as a pattern of geographical parthenogenesis in the tropics. Apomictic tetraploids of *Z. mackayi* also have larger distributions compared to diploids, but the inability of this species to self-pollinate led Gomes et al. (2018) to attribute greater reproductive success of tetraploids to polyploidy consequences on physiological traits, as tetraploids occur in areas of marked temperature seasonality. Our results from this study point to an additional putative effect of polyploidy on the reproductive success of *Z. mackayi*.

The outcome of self-pollinations performed in *Z. mackayi* showed higher reproductive success of tetraploids compared to other cytotypes, as indicated by fruit set (66.2% tetraploids, 57.1% triploids, 33.3% diploids). While high fruit abortion in triploids is an expected outcome of meiotic irregularities caused by unbalanced chromosome number (Comai 2005), the elevated frequency of fruit abortion in self-pollinated diploids suggests the occurrence of a pre-zygotic incompatibility system. The analyses of ovule differentiation in aborted diploids indicated arrestment of ovule development followed by placental degeneration. As no abnormalities were observed during pollen germination and pollen tube development to the ovary, we propose a late-acting self-incompatibility (LSI) mechanism is acting in diploid individuals of *Z. mackayi*. The distinction between LSI and inbreeding depression is challenging, but LSI mechanisms are known to cause fruit abortion at the same developmental stage for a given species, while inbreeding depression acts in different stages of a plant´s life cycle, mainly along embryo development (Seavey and Bawa 1986; Gibbs 2014). In addition, population genetic studies of *Z. mackayi* discarded the occurrence of inbreeding in any cytotype (Moura et al. 2020).

Polyploidization events are commonly related to the breakdown of self-incompatibility system (Barringer 2017). *Z. mackayi* apomictics are not reproductive autonomous like other apomictics from the Brazilian savanna. However, higher fruit set of polyploid apomictics compared to diploid sexuals may significantly increase reproductive success, as reproduction in deceptive orchids like *Z. mackayi* is severely limited by low pollinator visitation frequencies (Neiland and Wilcock 1998; Campacci et al. 2017). The larger geographical range of tetraploids compare to diploids of *Z. mackayi* is likely a result of combined effects of polyploidy, high fruit set and polyembryony, which allows for higher seed production and plasticity compared to other cytotypes. These results suggest ecological and evolutionary consequences of apomixis may be more diverse in tropical biomes than previously described for temperate species (Hörandl 2006). More data is needed, especially considering herbaceous species, to fully understand the role of apomixis in range expansions of species in tropical ecosystems.

Declarations

The authors have no competing interests to declare that are relevant to the content of this article.

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AUTHOR CONTRIBUTION STATEMENT

GVC, JLSM, SK conceived the study. GVC, MFA, JLSM collected and analyzed the data. All authors wrote and approved the manuscript.

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Tables

Table 1

Developmental stages of ovule and embryos of flowers and fruits of diploids, triploids and tetraploids Zygopetalum mackayi. Time intervals are indicated as days after pollination. AEPs = adventitious embryo precursor cells

developmental stage	diploid	triploid	tetraploid
placental proliferation and ovule initiation differentiation	1–12	1–12	1–12
pollen tubes arrive in ovary	13	13	13
archesporial cell differentiation	20	20	20
megaspore mother cell differentiation	22	30	22
meiosis and megaspore dyad/tetrad formation	24	38	24
degeneration of the three micropylar megaspores; differentiation of AEPs in the nucleus of polyploids	24	40	32
mature embryo sac	60	45	40
egg cell fertilization	64	60	42
zygote and endosperm formation	66	60	44
endosperm degeneration	70	64	46
first divisions of the zygote and AEPs in polyploids young globular embryos and suspensor differentiation	70	66	48
seed coat tegmen begin to degenerate; seed testa expand and lignify differentiation of protoderm in the embryo	82	78	66
mature embryos and interruption of their mitotic activity; mature seeds	102	90	86
seed coat with single thin layer (testa); suspensor degeneration	134	98	100

Figures

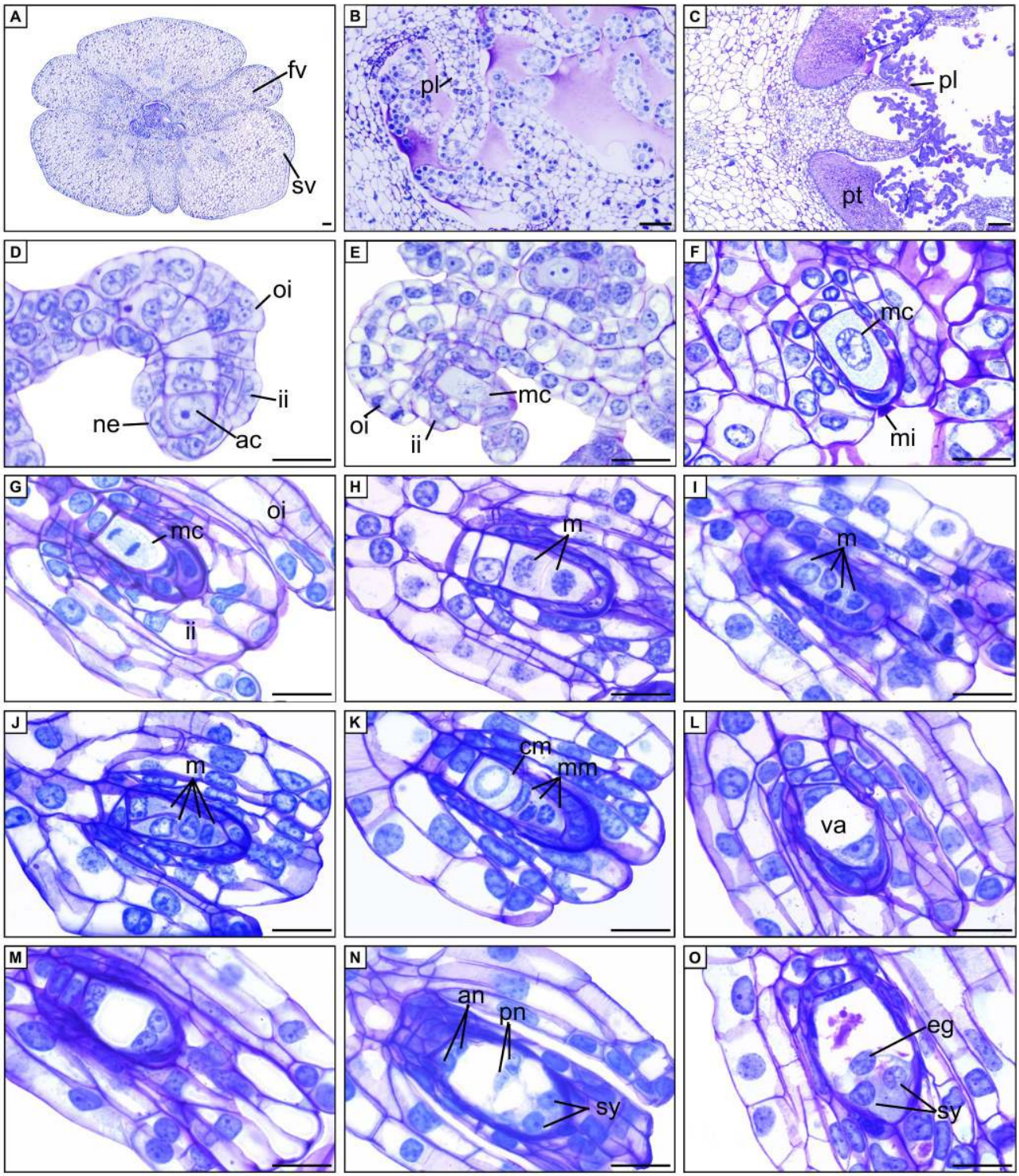


Figure 1

Longitudinal sections of ovules of *Zygopetalum mackayi* Hook. **A** Overview of the ovary of the anthetic flower. **B** Placenta with ovules at the beginning of development. **C** Pollen tube at placenta 20 days after pollination. **D** Differentiation of the initial archesporial cell and formation of the integuments. **E** Megaspore mother cell. **F** Megaspore mother cell and integuments. **G** Megaspore mother cell during meiosis I. **H** Megaspore dyad. **I** Linear tetrad of megaspores. **J** Inverted T-shaped tetrad of megaspores.

K Chalazal megaspore expanding and degenerating micropylar megaspores. **L** Binucleate megagametophyte. **M**Tetranucleate megagametophyte. **N** Mature megagametophyte. **O** Synergids and egg cell. ac= archesporial cell; an= antipodes; cm= chalazal megaspore; eg= egg cell; fv= fertile valve; ii= inner integument; m= megaspore; mc= megaspore mother cell; mi= micropyle; mm= micropylar megaspores; ne= nucellar epidermis; oi= outer integument; pl= placenta; pn= polar nuclei; pt= pollen tube; sv= sterile valve; sy= synergids; va= vacuole. A, B, E, G, I, J, L, M, O= tetraploid cytotype; D, H= triploid cytotype; C, F, K, N= diploid cytotype. Scale bars: A-C= 20µm; D-O=10µm

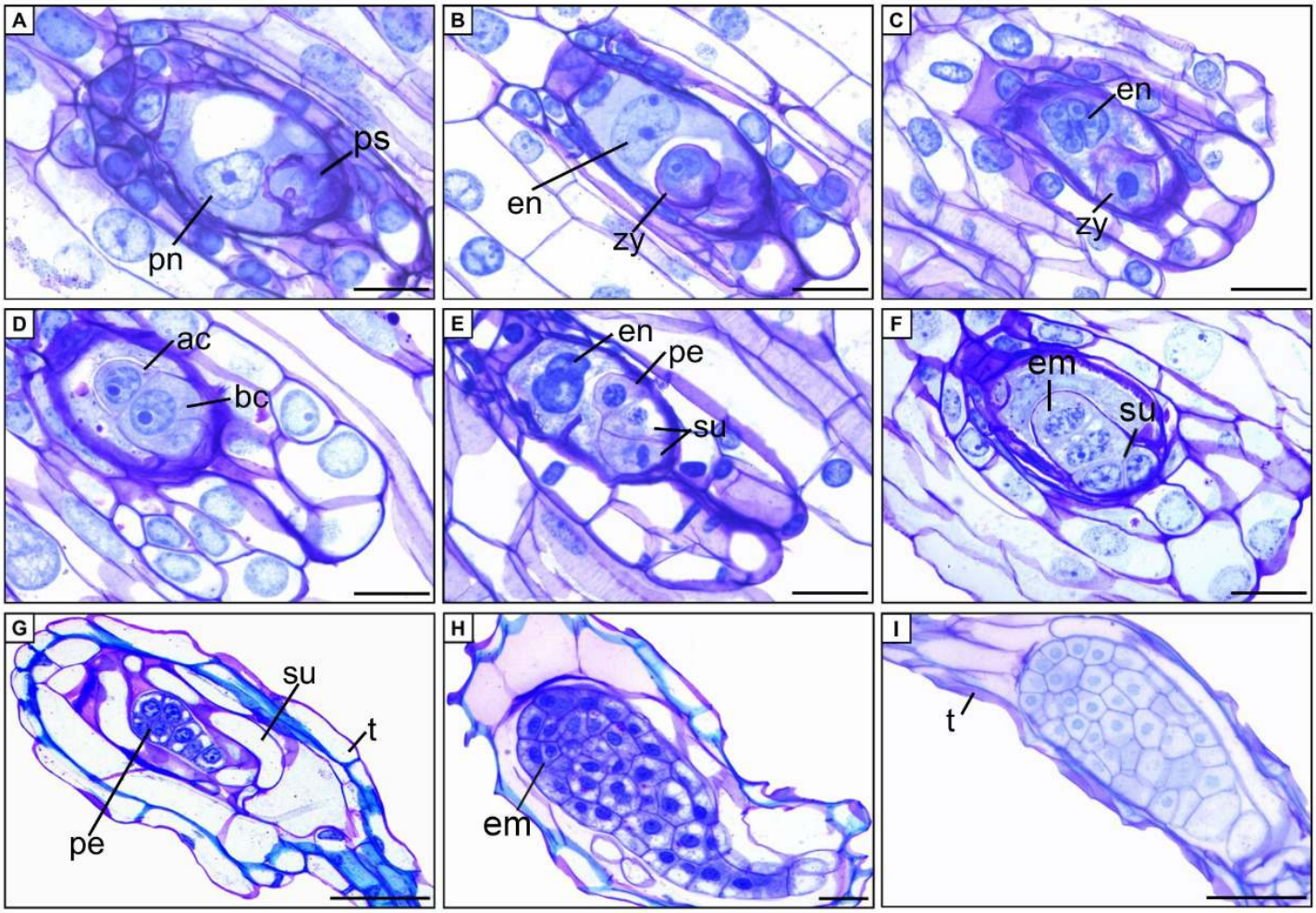


Figure 2

Longitudinal sections of ovules and seeds of *Zygopetalum mackayi* Hook. **A** Penetrated synergid. **B** Zygote and primary endosperm nucleus. **C** Zygote and nuclear endosperm. **D** Embryo with two cells. **E-G** Embryo and suspensor. **H-I** Mature seed. ac= apical cell; bc= basal cell; em=embryo; en= endosperm; pe= pro-embryo; pn= polar nuclei; ps=penetrated synergid; t= testa (seed coat); su= suspensor; zy= zygote. A-B, D, F, H= tetraploid cytotype; E= triploid cytotype; C, G, I= diploid cytotype. Scale bars: A-F= 10µm; G-I= 20µm

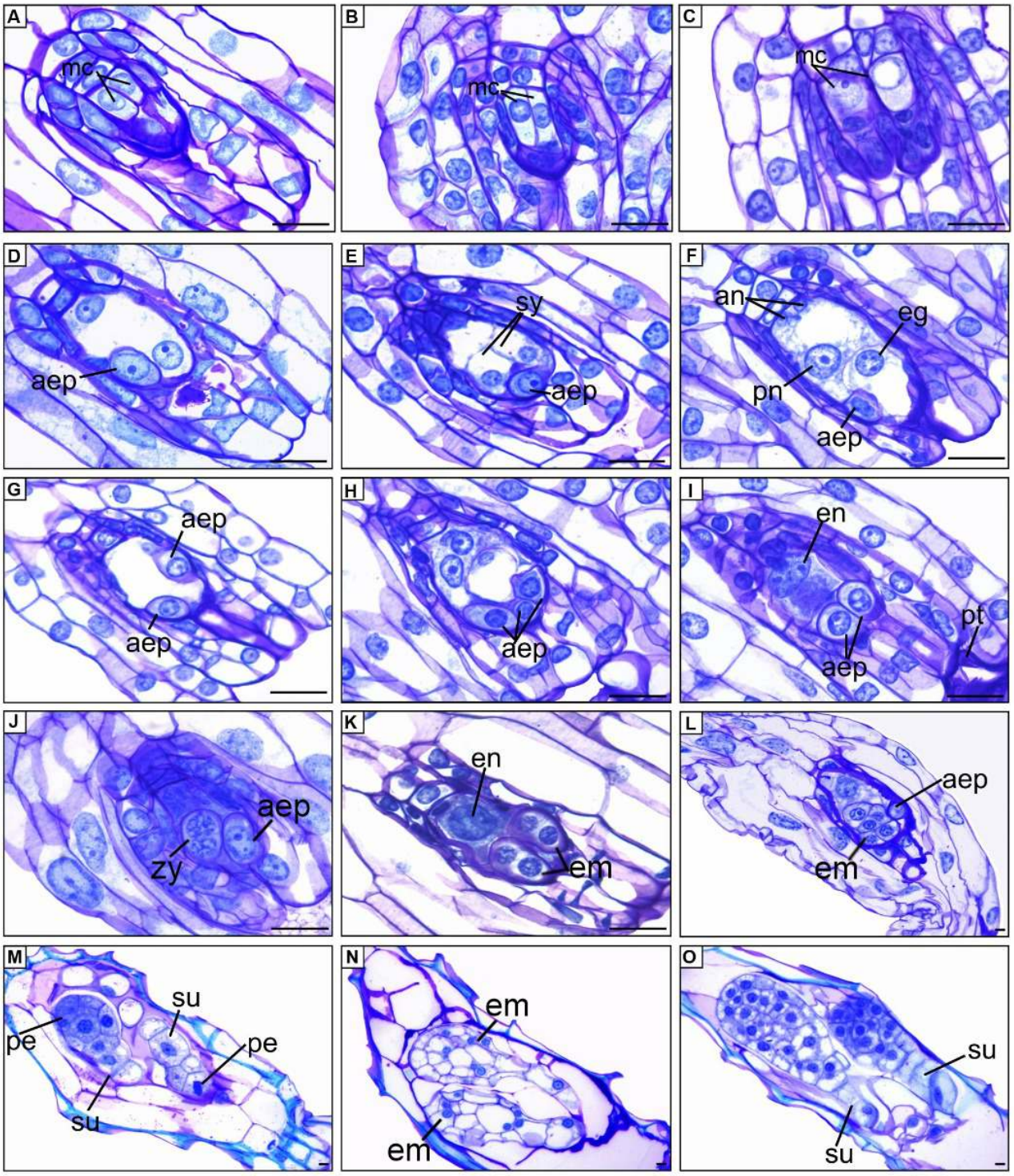


Figure 3

Longitudinal sections of ovules and young seeds in *Zygopetalum mackayi* Hook. **A-C** Differentiation of two megaspore mother cell in the same ovule. **D-E** Adventitious embryo precursor cell (AEP) in the integument of the micropylar region. **F** AEP and egg cell. **G**Two AEPs in the micropylar region. **H** Three AEPs in the micropylar region. **I** AEPs and endosperm. **J** Zygote and AEP. **K** Two zygotes and endosperm. **L** Five cell embryo and AEP in the the micropylar region. **M-O.** Sexual and adventitious embryo. aep=

adventitious embryos precursor cells; an= antipodes; eg= egg cell; em= embryo; en= endosperm; mc= megaspore mother cell; pe= pro-embryo; pn= polar nuclei; pt= pollen tube; su= suspensor; sy= synergids, zy= zygote. C, D, F, H, I, K-O = tetraploid cytotypes; G, J= triploid cytotype; A, B= diploid cytotype. Scale bars: A-I = 10µm, J-O= 20µm

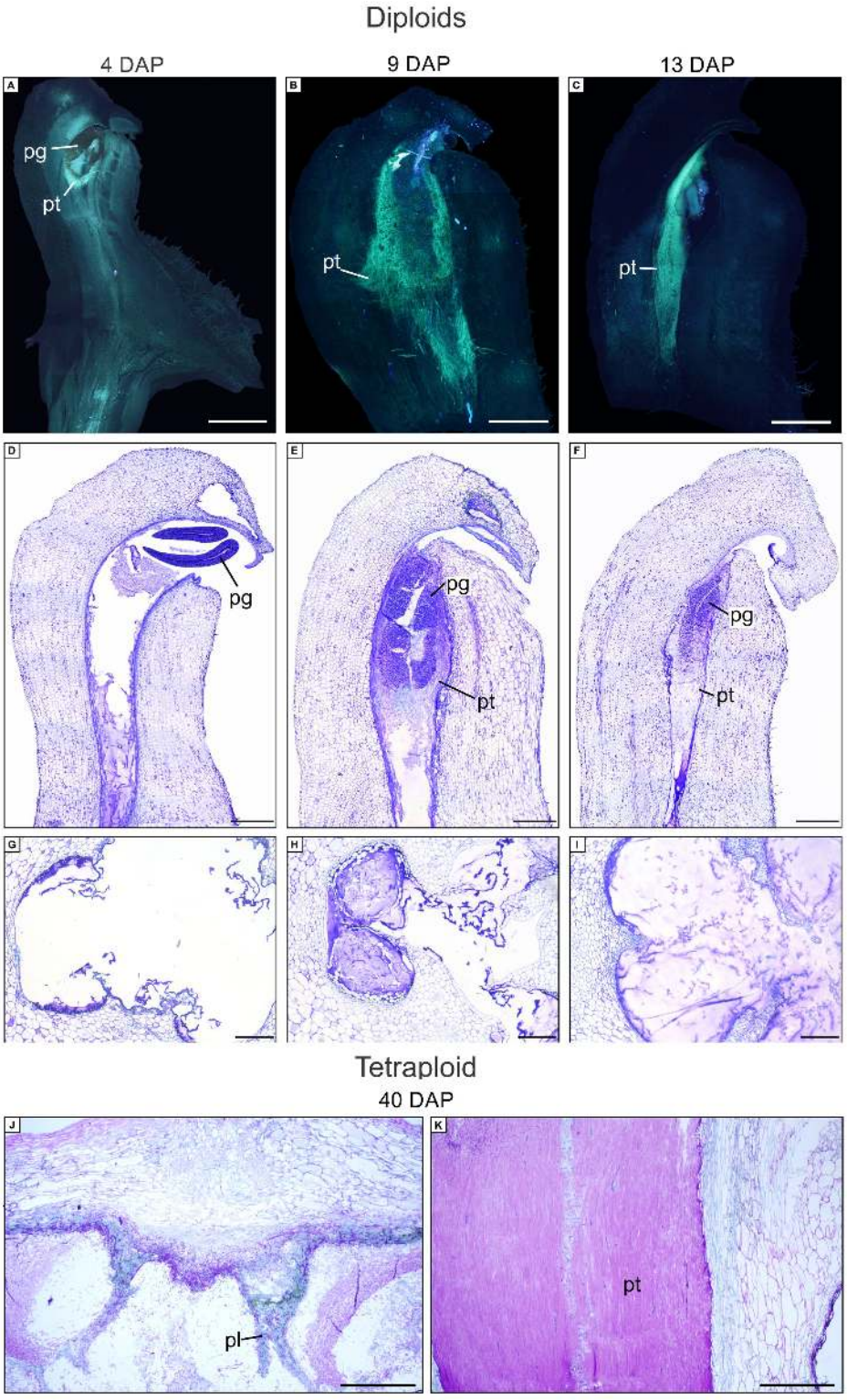


Figure 4

Longitudinal section of column of *Zygopetalum mackayi* Hook., showing pollen tube growth after self-pollination. **A-C**epifluorescence microscope image; **D-K** light microscopy image. **A, D** Pollen grain germination four days after pollination. **B, E** Growth of pollen tubes in column nine days after pollination. **C, F** Pollen tubes in ovary 13 days after pollination. **G** Degenerated placenta nine days after pollination. **H**. Degenerated placenta 13 days after pollination. **I** Degenerated placenta 40 days after pollination. **J** Degenerated placenta 40 days after pollination. **K** Pollen tubes growth inaborted fruits 40 days after pollination. pg= pollen grain; pl= placenta; pt= pollen tube. A-I= diploid cytotype; J-K= tetraploid cytotype. Scale bars: A-F= 200µm; G-I= 100µm

Supplementary Files

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- TableS1.docx