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Developmental analyses of the phyllomorph formation in the rosulate species *Streptocarpus rexii* (Gesneriaceae)

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Abstract. Although some species of *Streptocarpus* (Gesneriaceae) do not possess a layered shoot apical meristem (SAM), but three individual meristems, the basal meristem (BM), the petiolode meristem (PM) and the groove meristem (GM) on the petiolode from which additional phyllomorphs are formed. To gain insights into the processes involved, we examined the development of seedlings from germination to the formation of the primary phyllomorph in *S. rexii*, a rosulate species. Our specific focus was to examine the relationship between the functional activity of the GM and meristematic activity, which was assessed by a combined analysis of toluidine blue staining of histological sections and the incorporation of BrdU into meristematic tissues. The results were integrated into 3-D graphics, which suggests a complex spatial and temporal interaction within the GM. The significance of our observations is discussed and compared to the SAM observed in most other angiosperms.

Key words: *Streptocarpus rexii*, Gesneriaceae, leaf formation, shoot apical meristem, groove meristem.

In most angiospermous plants, the upper part of the plant body is formed from a strictly structured, and layered shoot apical meristem (SAM) (Poethig 1997), in which interactions between stem cell activities in the central zone (CZ) and cell division activities of the surrounding peripheral zone (PZ) are observed (Meyerowitz 1997, Clark 2001). Recent studies revealed that molecular interactions between stem-cell maintaining activities in the CZ and differentiation-directing signals in the PZ play an important role in organizing lateral organs including leaves (Bowman and Eshed 2000). In addition, the formation of leaf primordia is regulated by other intrinsic, external factors and internal factors such as plant hormones (Reinhardt et al. 2000, 2003). Such studies of leaf development have mostly been carried out on model plants, such as *Arabidopsis thaliana*.

However, there are certain members of Gesneriaceae in which an apparent embryonic SAM cannot be recognized (Jong 1970, Tsukaya 1997, Imaichi et al. 2000, Nishii et al. 2004, Mantegazza et al. 2006); seedlings of these plants exhibit 'anisocotyly', whereby one of the two cotyledons continues growth after germination to form a macrocotyledon, which remains the sole leaf organ produced throughout the life history in 'unifoliate' species of *Streptocarpus* and *Monophyllaea*. Nonetheless, towards the end of their life cycle they bear inflorescences at the base of the lamina

(Hilliard and Burtt 1971, Weber 1975). Such anisocotyly is ubiquitously observed in Old World Gesneriaceae (Möller and Cronk 2001). The genus *Streptocarpus* is intriguing, since it harbours acaulescent (unifoliate & rosulate), but also caulescent species, the latter possessing a SAM. Jong and Burtt (1975) described in detail the ontogenesis of the rosulate *Streptocarpus fanniniae*, introducing the 'phyllomorph' concept, a leaf construct composed of a lamina and 'petiolode', a structure with a dual function of petiole and stem. In *Streptocarpus* seedlings three regions were identified, the basal meristem (BM), petiolode meristem (PM), and the groove meristem (GM), that are ascribed to have meristematic activities (Jong and Burtt 1975, Jong 1978). The BM is thought to be responsible for the expansion of the lamina, while the PM extends the midrib and petiolode. In 'rosulate' species of *Streptocarpus*, leaves are formed successively from the 'groove meristem' (GM) situated on the 'petiolode', that is apparently not homologous to a SAM (Jong and Burtt 1975, Jong 1978, Harrison et al. 2005). Harrison et al. (2005) demonstrated that *knox*-like genes are expressed upon the formation of the GM. This implies that there exists a degree of homology between the meristematic regions of acaulescent *Streptocarpus* species and those of 'ordinary' angiosperms.

Characterization of the underlying cellular mechanisms of meristem formation should deepen our understanding of the formation of lateral organs in angiosperms in general because the unique feature should share common feature of other angiosperms. To this end, we examined the ontogenetic formation of the first leaf in the rosulate *Streptocarpus rexii*, with particular focus on the relationship between the GM and the initiation of cell divisions, as measured by BrdU incorporation into the tissues.

Materials and methods

Plant materials. Seeds of *Streptocarpus rexii* Lindl. were kindly supplied by Dr. M. Möller, Royal Botanic Garden Edinburgh (Edinburgh, UK). The seeds were sterilized as described in Nishii et al. (2004), and sown on a culture medium consisting of 30 % strength MS medium (Murashige and Skoog 1962), solidified with 0.8 % agarose in 9 cm plastic Petri dishes. Plants were cultured in a growth chamber at 23°C under a regime of 18 h light (80 μmol m^{-2} s^{-1}) and 6 h dark.

Morphological analyses. To examine phyllomorph formation patterns, plants were observed under a dissecting microscope SZX9 (Olympus Optical Industries Co., Tokyo, Japan), and photographed. For observations under a scanning electron microscope, samples were prepared using the critical point drying method as described in Nishii et al. (2004). The samples were gold ion-sputter coated and observed using a SEM S-2250N (Hitachi) at the University Museum of the University of Tokyo. The mold case technique was also used as described by Imaichi et al. (2000).

For the observation of internal structures, samples were fixed in a solution of 4 % paraformaldehyde, 1 % glutaraldehyde, in 20 mM sodium cacodylate buffer (pH 7.2), embedded in Technovit 7100 resin (Heraeus Kulzer GmbH & Co., KG, Wehrheim, Germany), and sections prepared as described in Kuwabara et al. (2001). Samples were sectioned to 3 μm in thickness, and stained with Toluidine Blue (TBB) at 30 μm intervals. Samples were observed under a microscope (BX51, Olympus), and photographed.

Assessment of meristematic activity. After incubation of seedling in BrdU (Nishii et al. 2004), sections of 3 μm in thickness were prepared from tissues embedded in Technovit 7100. Alternating sections in a 30 μm interval were analysed for BrdU incorporation or stained with TBB and their photographic images were taken with a digital camera DP70 (Olympus). TBB staining was used to pinpoint the position of meristematic regions, particularly the GM, as it was found to be characterised by small cells that deeply stained blue with TBB (Jong 1978). BrdU incorporation into the cells was assessed under a fluorescence microscope (BX51, Olympus) after staining using an indirect fluorescence immunostaining method as described in Nishii et al. (2004). Cells whose nuclei were stained with a fluorochrome-labelled antibody were considered meristematically active.

Construction of 3-D graphics. To relate the position of meristems with meristematic activity, the above-mentioned photographic images were transferred to a computer and the images of serial sections of one plant were superimposed sequentially using Photoshop 6.0 (Adobe Systems, CA, USA). Positions of the cells and section contours were measured in reference to an appropriate fixed position. The obtained data sets were plotted in a 3-D scatter graph using the graphic program Origin 7.5 (Origin Lab., MA, USA).

Results

Seedling growth of *S. rexii* on agar. After germination two equally sized cotyledons (isocotyly) were observed (Fig. 1A). About two weeks later one cotyledon became visibly larger (anisocotyly). Subsequently, the first, primary phyllomorph was formed near the base of the macrocotyledon (Fig. 1B). Further phyllomorphs were generated from near the base of preceding phyllomorphs. On agar, a quick succession of further phyllomorphs was observed in *S. rexii*, resulting in a rosulate plant (Fig. 1C, D). In addition, a new phyllomorph (P1') was formed in the axil of the macrocotyledon, additionally to the primary phyllomorph, resembling lateral branching (Fig. 1D). The formation of axillary phyllomorphs in successive phyllomorphs was also observed at later stages of plant development.

Ontogeny of phyllomorph formation in *S. rexii*. To examine the ontogenetic process of the formation of the primary phyllomorph in more detail, seedlings of different ages were observed by SEM. After the establishment of anisocotyly, a group of small, presumably meristematic cells were observed on the petiolode near the base of the lamina of the macrocotyledon (Fig. 2A, an arrow). This represents the GM (Fig. 2A) from which the first phyllmorph will develop. We refer to this phase as the flat GM stage (Fig. 2A). Subsequently the GM developed into a bulge. This stage we termed bulged GM stage (Fig. 2B, b).

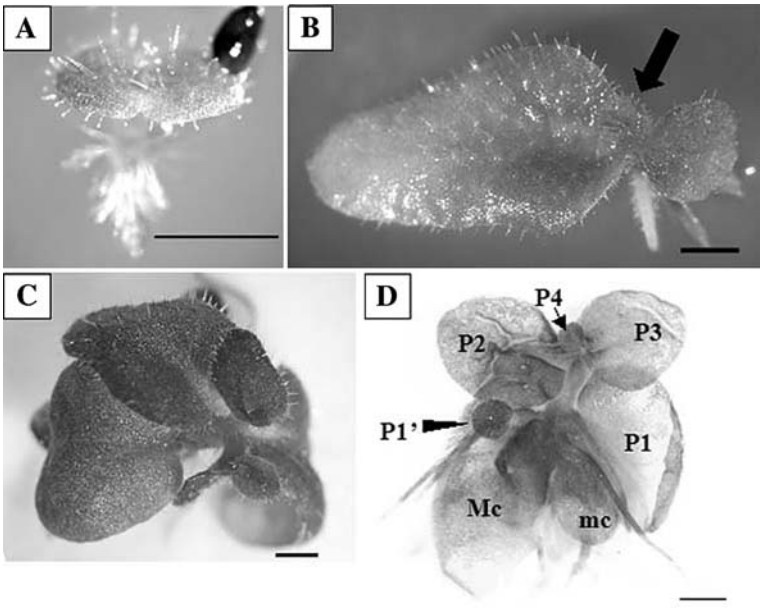


Fig. 1. Ontogenetic process of phyllomorph formation in *S. rexii* on agar. **A** Isocotylous stage. **B** Anisocotyous stage; arrow: the primary phyllopmorph. **C** Reiteration of phyllomorphs result in a rosulate plant. **D** Succession of phyllomorph development indicated by increasing numbers P1 to P4. Mc: macrocotyledon, mc: microcotyledon, P1: the primary phyllomorph, P2: the second phyllomorph, P3: the third phyllomorph, P4: the forth phyllomorph, P1': axillary phyllomorph (arrowhead). Scale bars = 1 mm

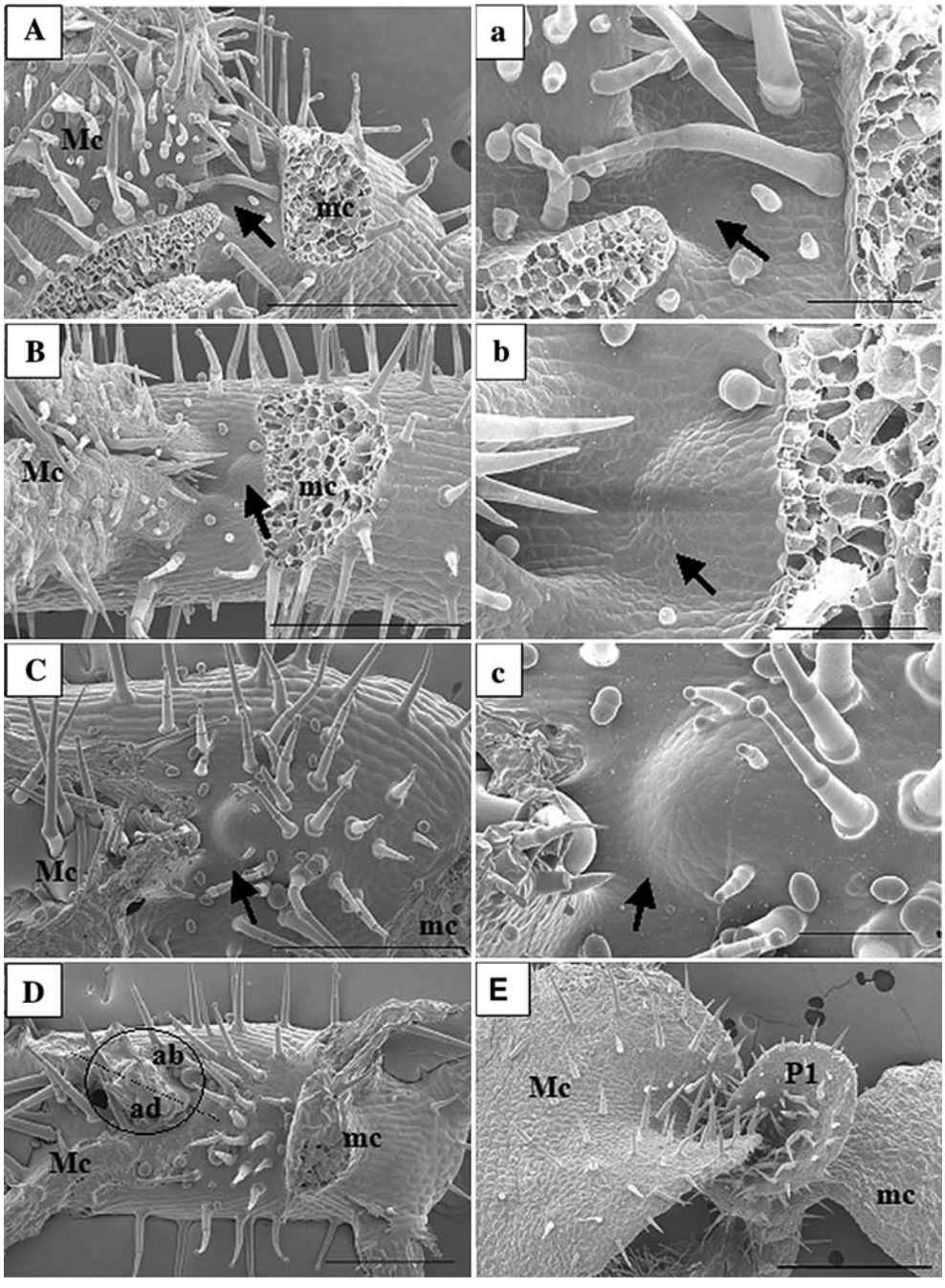


Fig. 2. Scanning electron micrographs illustrating the ontogeny of the primary phyllomorph in *S. rexii*. **A, a** Flat GM stage. **B, b** Bulging GM stage. **C, c** Dome-shaped GM stage. **D** Leaf formation stage. **E** Larger primary phyllomorph of 6 weeks old seedling. In A to D the microcotyledon and part of the macrocotyledon was removed to reveal the GM and primary phyllomorph. **a, b, c** Magnified views of A, B, C. arrow: GM, circle (D) and P1(E): the primary phyllomorph, ab: abaxial and ad: adaxial side of lamina. Scale bars = 500 μm (A–D), 100 μm (a–c), 1 mm (E)

Fig. 3. Position of the groove meristem (GM) in *S. rexii* determined by histochemical staining with TBB. **A, B**. Isocotylous seedling shortly after germination. **A** Longitudinal section from the central region (0 μm). The small cells were observed on the base of cotyledons (arrowhead). **B** Longitudinal section 30 μm off-center. **D–F**. Transverse serial sections of an anisocotylous seedling in the flat GM stage. asterisks: BM, arrowhead: GM. **D** Section through the base of the lamina of the macrocotyledon (position 0 μm). **E** Section at 30 μm in front of the base of the lamina. **F** Section at 60 μm on the petiolode outside the GM. **H–J**. Transverse serial sections of an anisocotylous seedling in the dome-shaped GM stage. arrowhead: GM. **H, I** Sections at 0 and 30 μm showing an organized GM. **J**. Section at 90 μm outside the GM. **L-M**. Longitudinal serial sections of a seedling with primary phyllomorph. **L** Section through the center of the primary phyllomorph (0 μm). arrowhead: GM of primary phyllomorph, *BM*: basal meristem, *PP*: primary phyllomorph, *pp*: petiolode of primary phyllomorph, *pl*: lamina of primary phyllomorph. **M** Section 60 μm off centre. arrowhead: trace of the former GM. **C, G, K, N** Schematic illustration of directions (arrows) and positions (shading) of sectioning. *Mc*: macrocotyledon, *mc*: microcotyledon, *GM* (arrowhead): groove meristem, *PP*: primary phyllomorph. Scale bars = 100 μm

At the dome-shaped stage, glandular trichomes were observed in parts of the GM that will form the lamina of the primary phyllomorph (Fig. 2C, c). The emergence of trichomes seems to reflect the onset of cell differentiation and leaf identity within the GM and illustrates the position of the primary phyllomorph. Later in development the primary phyllomorph became more distinct in shape and the lamina appeared at an angle on the petiolode of the macrocotyledon (Fig. 2D), which we termed the leaf formation stage. This stage was very flexible in *S. rexii*; the primary phyllomorph does not necessarily develop opposite to the macrocotyledon (i.e. its adaxial side faces the macrocotyledon). In the seedling depicted in Fig. 2D the adaxial side of the first lamina developed not in a right but oblique angle to the petiolode (circle). In a population of seedlings observed in detail the direction of primary phyllomorph development was not fixed (data not shown).

Relationship between the location of meristems and meristematic activity in *S. rexii*. At the isocotylous stage after germination some small cells were observed between the two cotyledons but a structured SAM was lacking (Fig. 3A, arrowhead). At this stage of seedling development, cells at the base of both cotyledons were deeply stained with TBB (Fig. 3B), which suggests that they defined the extent of the BM. The meristematic identity of this region was confirmed by BrdU incorporation (Fig. 4A, B). Superimposition of TBB and BrdU data illustrated that the meristematic activity of the BM was higher than that of the GM cells at this stage. (Fig. 5A–C).

After the establishment of anisocotyly, a tunica-corpus-like structure was observed in the flat GM (Fig. 3D, E). The size of the GM had increased and was located at the base of the macrocotyledon. At this stage BrdU incorporation into the GM became notable (Figs. 4D, 5D–F). BrdU incorporation into the BM was limited to the macrocotyledon but extended into the vascular bundles of the petiolode that maybe linked to the PM (Fig. 4C–E).

At the dome-shaped GM stage, the growth of the GM became conspicuous, and the extent within the petiolode was indicated by the increased size of the cluster of cells deeply stained with TBB (Fig. 3H, I). A vascular strand additional to the central vascular strand of the petiolode developed to support the growing GM (Fig. 3I, J). Spatially, BrdU incorporation was concentrated in the BM and GM but also in its surrounding area, especially beneath the GM (Figs. 4F–H, 5G–I). Intriguingly, incorporation of BrdU within the petiolode was more evident than that onto the surface, but more diffuse (Fig. 5G, I). This is in line with the diffuse nature of the PM.

At the primary phyllomorph formation stage, some incorporation of BrdU into the

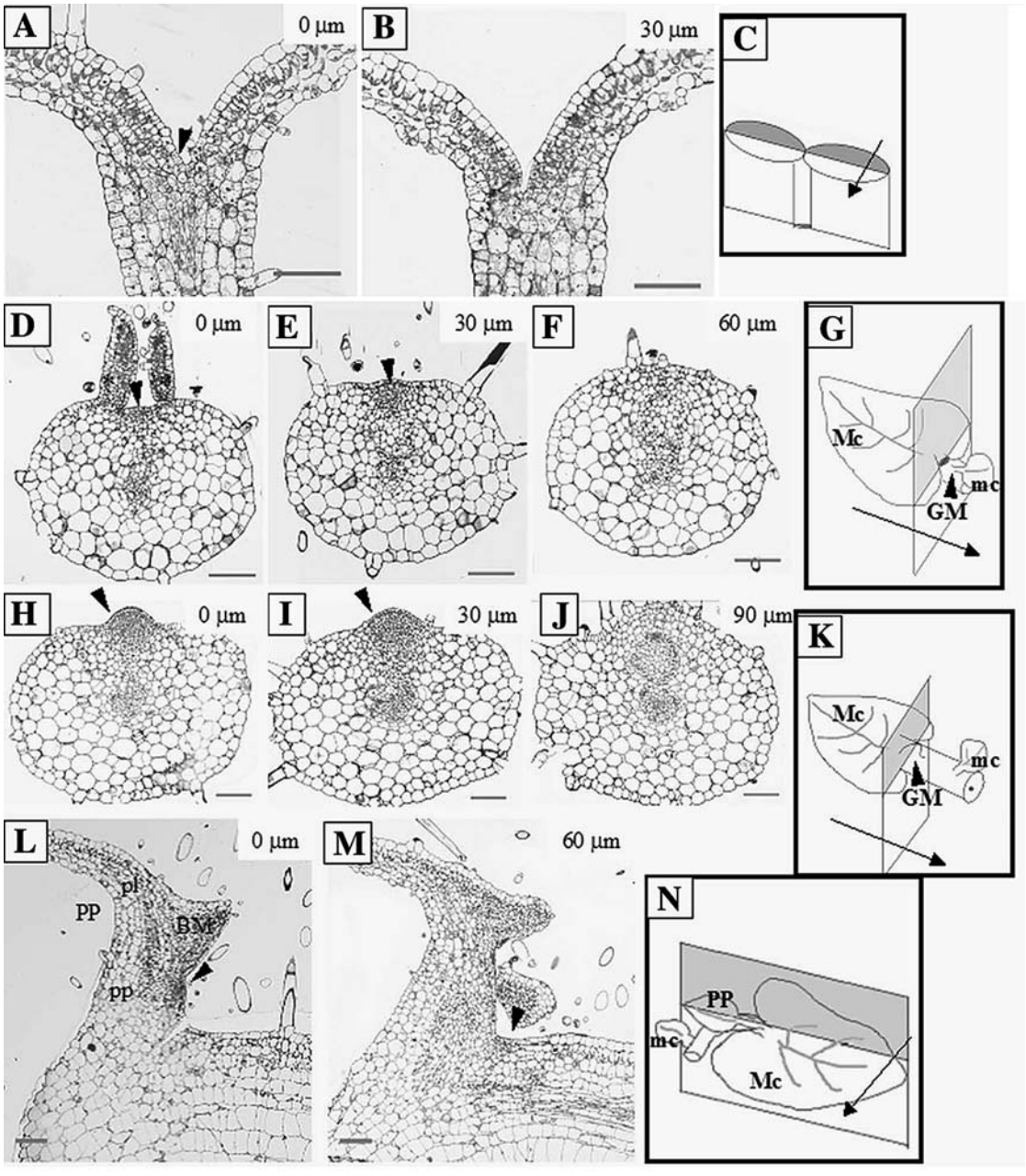
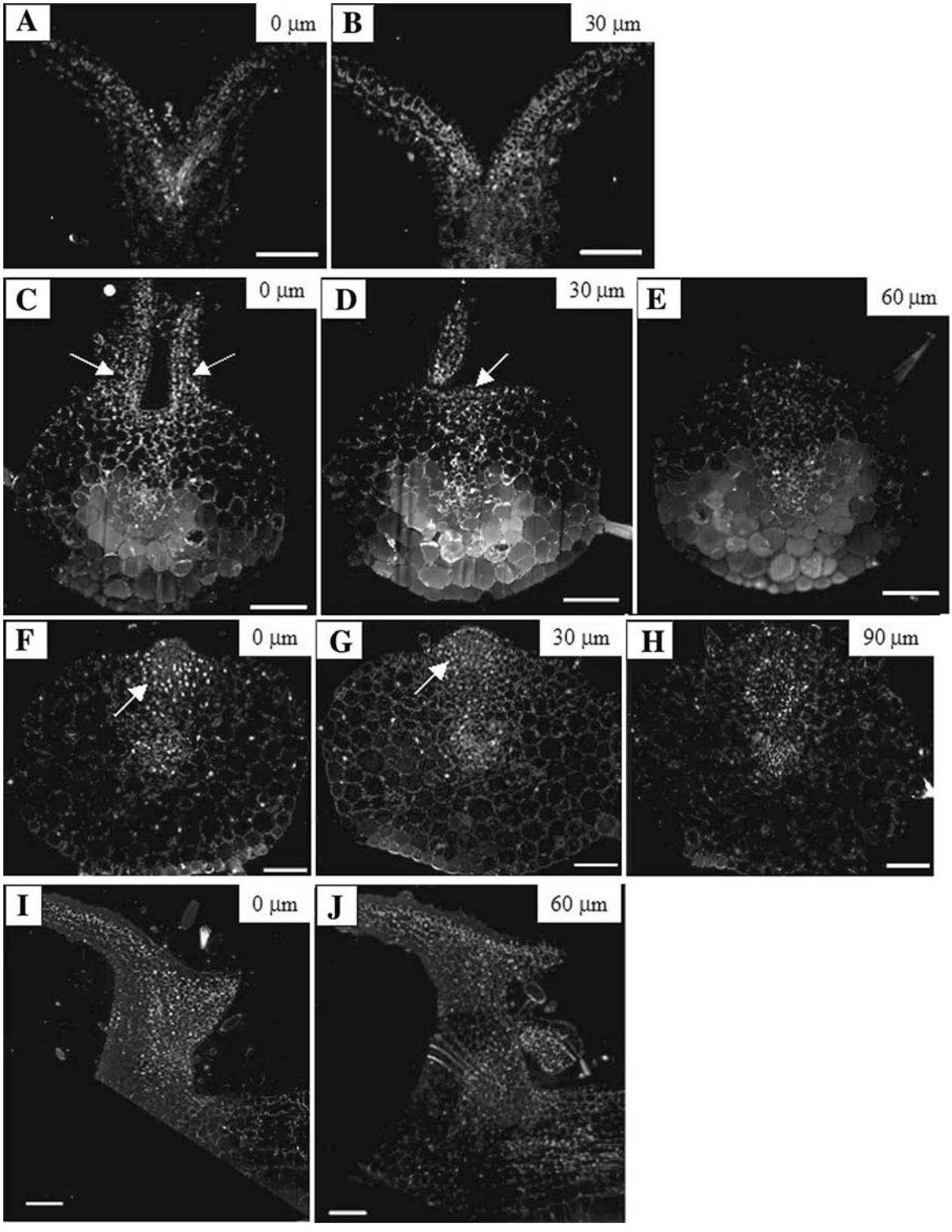


Fig. 4. Localization of the incorporation of BrdU into nuclei as revealed by fluorescence microscopy in seedlings of *S. rexii*. Cells whose nuclei emitting green fluorescence were at the S phase of the cell cycle. Each section corresponds to the adjacent section in Fig. 3. **A, B** Isocotylous seedling shortly after germination. **C–E** Anisocotylous seedlings at the flat GM stage. **C** BM activity was observed in the proximal region of the lamina (arrows). **D** Positive signals were also observed in the center of the GM (arrow). **F–H**. Anisocotylous seedling at the dome-shaped GM stage. Positive signals were observed in the region below the GM (arrows). **I, J**. Seedling at the phyllomorph formation stage. Scale bars = 100 μm



newly developing phyllomorph was observed (Figs. 4I, J, 5J, L), indicating its high meristematic activity. The proximal part of the lamina of the first phyllomorph was characterized by deep staining with TBB (Fig. 3L, M) and high DNA synthesis (Fig. 4I), defining the area of

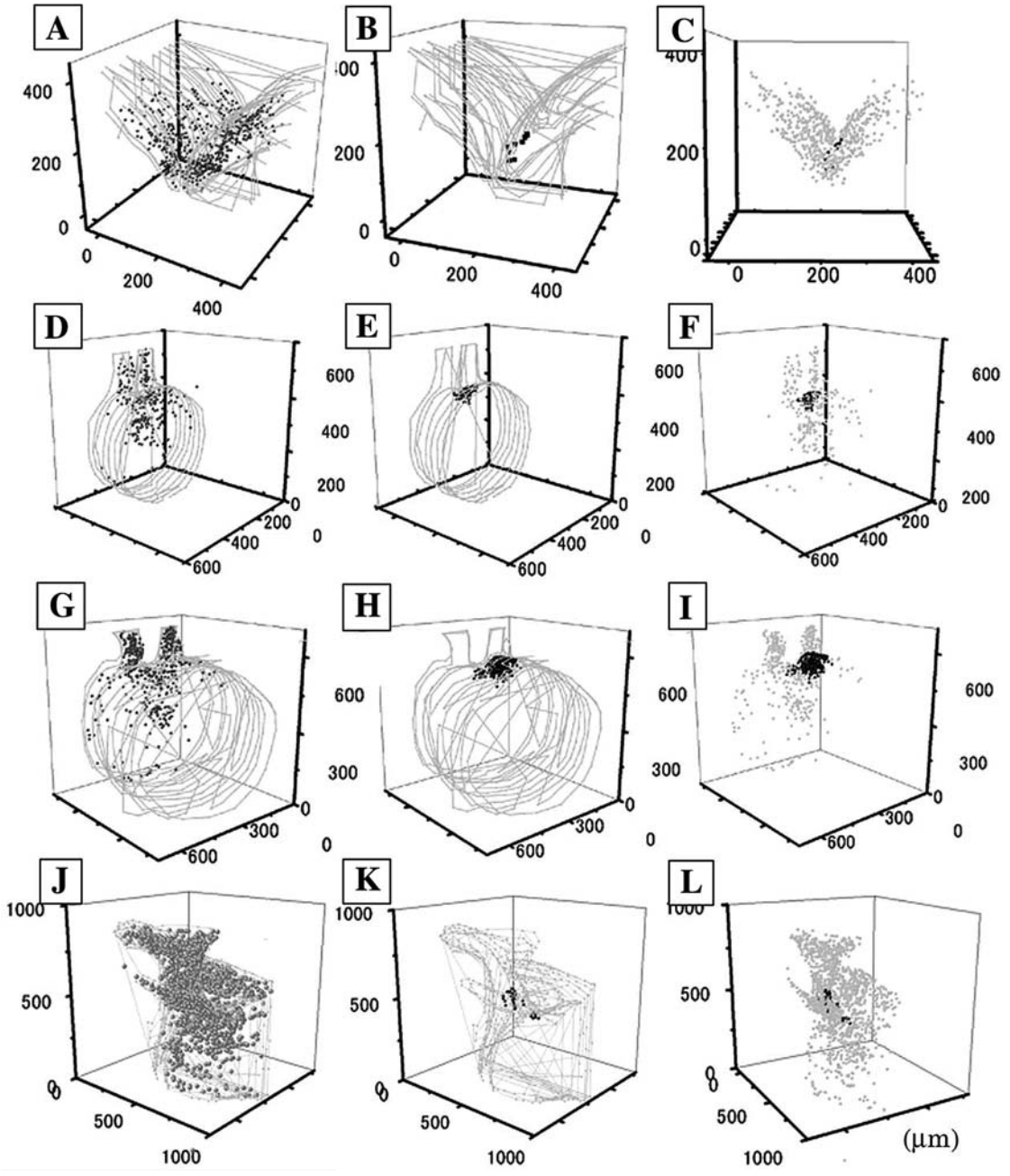


Fig. 5. Graphical illustration of TBB staining, BrdU incorporation and the superimposition of the two data sets. **A, D, G, J**. Plots of BrdU incorporation and the respective section contours. Light gray lines: section contours, dark gray circles: cells with BrdU incorporation. **B, E, H, K**. Plots of the position of GM cells, as identified by deep staining with TBB, and the section contours. light gray lines: section contours, black circles: GM cells. **C, F, I, L**. Plots of superimposition of the two data sets without section contours. black circles: GM cell, light gray circles: cells with BrdU incorporation. **A**–**C**. Isocotylous seedling. **D**–**F**. Anisocotylous seedling at the flat GM stage. **G**–**I**. Anisocotylous seedling at the dome-shaped GM stage, **J**–**L**. Seedling at the phyllomorph formation stage

its BM (Figs. 3M, 4I). GMs were found on the petiolode of the newly formed first phyllomorph and in the axil of the newly developed phyllomorph and the petiolode of the macrocotyledon (Figs. 3L, 5K, L).

Discussion

In this study the formation of the first 'foliage leaf' or phyllomorph was examined morphologically, anatomically and histochemically in *Streptocarpus rexii*, a rosulate species in Gesneriaceae. Our specific focus was to examine the establishment and activity of the GM in this process. This is particularly interesting as newly germinated seedlings in this genus lack a structured apical meristem. Although there have been several morphological and anatomical studies on this subject (Jong and Burtt 1975, Jong 1978, Harrison et al. 2005, Mantegazza et al. 2006), clarification of the interaction between the location and initiation of the GM and meristematic activity of the GM and surrounding tissue has been lacking. For simplification of comparisons, we classified the early phase of seedling development into five stages, the isocotylous stage, the flat GM stage, the bulging GM stage, the dome-shaped stage and the leaf formation stage.

The combination of localising meristematic regions, especially the GM region, as judged from TBB-staining and small cell size (Jong 1978), with their identification as meristematically active by mapping the incorporation of BrdU into newly formed cells, was useful in demonstrating the dynamism of cellular processes involved in the meristem formation in *Streptocarpus*. As expected the BrdU incorporation is limited to the proximal regions of both cotyledons at the isocotylous stage, where the BM resided, but the level of activity is surprising. The observed shift to the macrocotyledon and abandonment of meristematic activity in the microcotyledon at the anisocotylous stage is consistent with findings of Mantegazza et al. (2006) of a downregulation of the *knox*-like gene *SrSTM1* in the latter. However, the origin of the BM in the proximal region of the newly formed first phyllomorph must originate from parts of the GM, because of the spatial separation of the GM and BM of the macrocotyledon (Fig. 5I, L).

Jong and Burtt (1975) depict the PM as a region at the level of the GM transversely across the base of the midrib. The diffuse nature of this meristem was illustrated here by meristematic cells distributed in the surrounding regions of the GM (Fig. 5F, I). The onset of its activity coincided with the flat GM stage, and continued throughout macrocotyledon development and the first phyllomorph stage. Its activity is likely responsible for the displacement of the GM towards the base of the lamina of the macrocotyledon as suggested by Jong and Burtt (1975).

Displaying the two datasets in superimposed 3-D graphics suggests that the GM is initiated between the two cotyledons at the isocotylous stage in the position of the SAM in model plants. However, it first appears to be rather diffuse and not localised (Fig. 5C), but later appears more organised possessing a layered structure and displaced due to the activity of the PM (Figs. 3H, 5G). Thus, the GM is possibly spatially homologous to a SAM, but not developmentally and may represent an underdeveloped SAM. Elucidating the exact meristem by which the GM is initiated, from a diffuse cluster of cells or even a single 'stem' cell requires more detailed work.

Our study clearly revealed the dynamics of first phyllomorph initiation in *S. rexii* from a domed GM. The bulged GM later differentiated into a proximal and distal region, an indeterminate, meristematically persistent and determinate, lamina-forming part, respectively (Fig. 2C, c). This pattern of leaf formation in *S. rexii* was basically as described for excentric rosulate species of *Streptocarpus* (Jong 1978). It is not comparable to the leaf formation of adventitious shoots from callus (Skoog and Miller 1957). The indeterminate region of the GM seemed to split to form the GM of the newly formed

phyllomorph and an axillary-like meristem, remaining at the junction between the petiolode of the macrocotyledon and the petiolode of the first phyllomorph. This model would allow the development of axillary phyllomorphs, a 'branching', similar to the pattern reported by Jong (1978) for excentric species of *Streptocarpus*. This is also comparable to the properties of a typical SAM in model plants.

There may be extended homologies between a SAM of *Arabidopsis thaliana* and the GM in *Streptocarpus*. Certain aspects of the CZ (stem cell replenishment) and the surrounding PZ (cell division and differentiation of tissue) can be found in the GM, but vertically arranged (Fig. 2c), although dissimilarity may also be discernible, as the location of the GM appears to move, by the activity of the diffuse PM. To compare spatial similarity, functional characterizations of the GM with molecular probes of genes that have been identified in the SAM of model plants, such as *A. thaliana* and *Antirrhinum majus*, would be very revealing. We have now morphologically, anatomically and histochemically characterised the ontogenetic relationship between the three meristems in *Streptocarpus*. The next step would be their functional characterization using probes of different genes involved in meristem activities.

In conclusion, the combination of localising meristematic regions by TBB-staining with their identification as meristematic by the incorporation of BrdU into cell nuclei, proved to be a very powerful method in elucidating the spatial and temporal cellular processes involved in the formation of phyllomorph in *Streptocarpus*. Understanding of these processes increased our understanding of the cellular mechanisms underlying the intrinsic morphological lability in plants.

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