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**Positional cues regulate dorsal organ formation in the liverwort *Marchantia*
*polymorpha***

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Abstract

Bryophytes and vascular plants represent the broadest evolutionary divergence in the land plant lineage, and comparative analyses of development spanning this divergence therefore offer opportunities to identify truisms of plant development in general. In vascular plants, organs are formed repetitively around meristems at the growing tips in response to positional cues. In contrast, leaf formation in mosses and leafy liverworts occurs from clonal groups of cells derived from a daughter cell of the apical stem cell known as merophytes, and cell lineage is a crucial factor in repetitive organ formation. However, it remains unclear whether merophyte lineages are a general feature of repetitive organ formation in bryophytes as patterns of organogenesis in thalloid liverworts are unclear. To address this question, we developed a clonal analysis method for use in the thalloid liverwort *Marchantia polymorpha*, involving random low-frequency induction of a constitutively expressed nuclear-targeted fluorescent protein by dual heat-shock and dexamethasone treatment. *M. polymorpha* thalli ultimately derive from stem cells in the apical notch, and the lobes predominantly develop from merophytes cleft to the left and right of the apical cell(s). Sector induction in gemmae and subsequent culture occasionally generated fluorescent sectors that bisected thalli along the midrib and were maintained through several bifurcation events, likely reflecting the border between lateral merophytes. Such thallus-bisecting sectors traversed dorsal air pores and gemma cups, suggesting that these organs arise independently of merophyte cell lineages in response to local positional cues.

Keywords (4-6 keywords in alphabetical order)

Apical cell, Cell lineage, Clonal analysis, *Marchantia polymorpha*, Merophyte, Sectors

Introduction

Land plant development is characterized by apical growth, in which stem cells residing in the shoot apical meristem produce new tissues and organs, and changes in stem cell and apical function are linked to the origin of three-dimensional land plant forms and their subsequent diversification (reviewed in Harrison 2017; Moody 2020). Whilst bryophyte meristems are thought to have a single apical cell whose geometry and cleavage patterns determine organ position and plants' overall body plan (Harrison et al. 2009; Parihar 1967), lycophyte and fern meristems have one to a few apical cells (Harrison and Langdale 2010; Harrison et al. 2007; Sanders et al. 2011), and seed plants have multicellular meristems (Korn 2001, 2002; Poethig 1987; Poethig and Szymkowiak 1995). Apical cell daughters in non-seed plants are called 'merophytes' and proliferate to form clonally related cell groups (Douin 1925; Gifford 1983; Korn 1993). In mosses and leafy liverworts, the apical cells produce merophytes which divide to generate leaves or tissues such as epidermis and parenchyma (Crandall-Stotler 1980; Harrison et al. 2009; Parihar 1967; Ruhland 1924). Thus, merophytes serve as a repeating unit of shoot formation, and cell lineage is manifest in organ and tissue development. In contrast to other bryophytes, thalloid liverworts have flattened whole plant bodies in place of forming individual leaves (Crandall-Stotler 1981; Shimamura 2016), and patterns of thallus development and organogenesis are unclear.

The liverwort *Marchantia polymorpha* L. grows as a flattened creeping thallus that periodically bifurcates from the apical notch, which houses cuneate apical cell(s) (Fig. 1a, b; Shimamura 2016; Solly et al. 2017). The duration between consecutive notch bifurcation events is termed the "plastochron", and plastochron 1 covers the period between gemma germination and the first bifurcation (Fig. 1a; Solly et al. 2017). On the dorsal side of the thallus, an assimilation organ, the 'air chamber' (Fig. 1c), and a cup-shaped organ generating vegetative propagules, the 'gemma cup' (Fig. 1d), respectively develop repetitively or periodically. Histological and anatomical studies have shown that

air chamber formation begins with aperture formation at a distance from the apical cells, and then an intercellular space, the ‘air pore’ (Fig. 1b), forms between epidermal cells by cell separation (Fig. 1e; Apostolakos et al. 1982; Ishizaki et al. 2013). Though Apostolakos et al. (1982) concluded from observations of intercellular spaces at the corner of most epidermal cells in *M. paleacea* that air chambers have no distinct mother cell but develop from air pore cells surrounding the initial aperture, direct evidence supporting their conclusion is lacking. In contrast to air pores, gemma cup primordia are evident a few cells away from the apical cells in *M. polymorpha* (Fig. 1f; Barnes and Land 1908) suggesting that dorsal merophytes contribute to gemma cup development. Whether dorsal merophytes are the sole contributor, or other merophytes also contribute to gemma cup development is unclear.

This lack of clarity around dorsal organogenesis is inherent in histological and anatomical approaches which capture static snapshots of a series of dynamic developmental processes, and the difficulties in distinguishing merophyte borders in fully developed tissues. In this study, we used clonal analysis to label cell lineages (Poethig 1987), and found that both air chambers and gemma cups are formed across merophyte boundaries, suggesting that positional cues regulate organogenesis in the liverwort *M. polymorpha*.

Materials and Methods

Plant materials and growth condition

Male accessions of *M. polymorpha*, Takaragaike-1 (Tak-1) were used as wild-type plants. *M. polymorpha* was cultured on half strength Gamborg’s B5 medium containing 1% agar under 50-60 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ continuous white fluorescent light at 22 °C.

Plasmid construction and transformation

Plasmid pMpGWB337tdTN-GUS was generated by LR recombination between

pMpGWB337tdTN (Sugano et al. 2018) and pENTR-gus (Thermo Fisher Scientific, Waltham, MA, USA) using LR Clonase II (Thermo Fisher Scientific) and used for *Agrobacterium*-mediated transformation of Tak-1 thallus (Kubota et al. 2013). *pro*MpSYPI3B:*mTurquoise2*-MpSYPI3B vector was generated as described in Kanazawa et al. (2016). An *mTurquoise2*-coding sequence was amplified with a primer set, *mTurquoise2_BamHI_IF_Fw* (CCCCTTCACCGGATCATGGTGTCTAAGGGTGAGGAAC) and *mTurquoise2_BamHI_IF_Rv* (GCTGCCGCCGGATCCTTTGTAAAGCTCATCCATTCCG), and then inserted into the BamHI-digested *pro*MpSYPI3B:MpSYPI3B entry vector (Kanazawa et al. 2016) using In-Fusion HD Cloning System (TaKaRa, Shiga, Japan). The resultant entry vector was then introduced into pMpGWB101 (Ishizaki et al. 2015) by LR recombination, and then used for *Agrobacterium*-mediated transformation of pMpGWB337tdTN-GUS plant thallus (Kubota et al. 2013).

SEM imaging

SEM images were taken with 21-day-old Tak-1 thalli using TM3000 (Hitachi High Technologies, Tokyo, Japan).

Clonal analysis and fluorescence observation

Cre induction on mature gemmae was performed as follows: gemmae on a plate were treated with 3 μ L of a solution containing 5 μ M DEX, air dried for 10 to 20 minutes, and subjected to heat shock by incubating the plate in a 37 °C air incubator for various periods. Cre induction on thalli was performed as follows: 8-day-old thalli were vacuum-infiltrated in a solution containing 5 μ M DEX, transplanted onto agar media, air dried for 10 to 20 minutes, and then heat-shocked in a 37 °C incubator for 35 to 50 minutes.

Sector-formed thalli were embedded in 6% agar block and sectioned into ~200-

µm-thick slices with LinearSlicer Pro 7(DOSAKA EM, Kyoto, Japan). tdTomato, mTurquoise2, and bright field images in mature gemmae, 1-day-old gemmalings, 6-day-old thalli, and sliced samples were captured with fluorescence microscope BZ-X710 (Keyence, Osaka, Japan) and merged after equal adjustment of contrast and brightness for visibility by the BZ-X Analyzer (Keyence). Thalli were observed with fluorescence dissection microscope Leica M205 C (Leica, Wetzlar, Germany). Obtained images were manually merged by using ImageJ (<https://imagej.nih.gov/ij/>) after contrast and brightness were adjusted for visibility. Contribution of tdTomato-positive and -negative regions to a gemma cup was evaluated by counting the number of serrations existing in each region.

Histochemical assay for GUS activity

Histochemical assay for GUS activity was performed as described previously (Ishizaki et al. 2012).

Results

Exploitation of clonal analysis system

To determine how dorsal structures develop, we first established a Cre-*loxP* based clonal sector induction system (Fig. 2a). A construct was designed to constitutively express a floxed β -glucuronidase (*GUS*) gene under the *M. polymorpha* *EF1 α* promoter (*proMpEF1 α*), and conditionally express a Cre-recombinase/glucocorticoid receptor (GR) fusion protein under a *M. polymorpha* heat-shock promoter, *MpHSP17.8A1* (Fig. 2a; Nishihama et al. 2016). The rationale for sector induction was that, following heat shock and dexamethasone (DEX) treatment, the Cre protein would excise the *GUS* gene allowing expression of a nuclear targeted red fluorescent protein, tdTomato-NLS, under *proMpEF1 α* (Sugano et al. 2018). Two elements to control conditional expression of the *Cre* gene were included to avoid leaky excision of the floxed region. This system was

expected to mark clonal sectors negatively by the absence of GUS staining or positively by the presence of red fluorescent signal (Fig. 2b).

Clonal analysis requires the production of many sectors induced randomly at different stages of development, and there is therefore an optimal frequency of sector induction. Whereas a low frequency of induction generates too few sectors to generate sufficient data for analysis, a high frequency of induction can induce overlapping sectors, becoming uninformative. To optimise the frequency of sector induction and analyse organogenesis in gemmalings and thalli, we first treated gemmae with a solvent control solution or a solution containing 5 μ M DEX, and then heat-shocked gemmae for different periods of time at 37 °C. We subsequently examined gemmae by fluorescence microscopy. The frequency of cells showing fluorescence in the gemma increased as the duration of the heat-shock increased in DEX-treated gemmae, but increased to a lesser extent in untreated gemmae such that sectors were absent in gemmae with no heat shock and present in almost all cells in gemmae that were heat-shocked for 80 minutes (Fig. 2c). A 30-40 minute heat-shock induced sectors at a moderate frequency that was compatible with the requirements of clonal analysis, and was thus used in further experiments, unless otherwise noted.

Lateral merophytes bisect thallus lobes

Cre induction in mature gemmae and subsequent plant development caused sector formation in various patterns. Some sectors bisected the thallus lobe (Fig. 3), spanning the dorsal to ventral axis (Fig. 3a), and such sectors persisted through several bifurcation events with well-defined sharp borders (Fig. 3b), consistent with a lateral merophyte origin (see merophyte sectors in Harrison et al. 2007; Harrison et al. 2009; Sanders et al. 2011 for comparison).

Air pores are formed independently of merophyte cell lineage

Observations of air chambers at sector junctions showed an overlap with the edge of some air chambers, but other air chambers were divided into fluorescence-positive and -negative regions (Fig. 4a). Whilst the former constituted approximately half of cases, amongst the latter, a pattern where the sector border was positioned in the centre of the air pore predominated, but in some cases sectors were off centre (Fig. 4b). Only small proportion of air chambers had sectors that did not cross the air pore (Fig. 4b). Observation in an early developmental stage revealed sectors that covered not only entire air chambers but also half air chambers with the sector border positioned in the centre of the air pore (Fig. 4c). These data suggest that air chambers can be formed either within a merophyte cell lineage or between merophyte cell lineages (see Fig. 7).

Gemma cups are a multi-lineage-comprised organ

Next, we observed gemma cups that had formed during the second to fourth plastochrons (Fig. 5a; Solly et al. 2017) on thalli with persistent bisecting sectors (Fig. 3b) and found that such gemma cups were also bisected by the sectors and sometimes contained both fluorescence-positive and -negative gemmae (Fig. 5b). Not only the wall, but also the floor of gemma cups (where gemma initials arise) were separated by such sectors (Fig. 5c). These results suggest that gemma cups are not derived from a single merophyte, but multiple merophytes. Contribution of a fluorescence-positive region (or conversely a fluorescence-negative region) to a gemma cup varied depending on the timing of its formation relative to notch bifurcation; those formed during the second and fourth plastochrons showed a range of contributions from 0 to 100%, while those formed during the third plastochron showed approximately 50% contribution (Fig. 5d). These results suggest that gemma cups initiate from two or more cells at the boundary between thallus lobes arising from lateral merophytes, that the process of bifurcation has some inherent variability, and that positional cues are important in gemma cup development.

Gemma cup initials are first recognizable a few cells away from the apical cells (Barnes and Land 1908), and the dorsal merophyte is believed to participate in gemma cup development. However, this idea has never been demonstrated experimentally. Because gemma cups are never formed during the first plastochron (Fig. 5a), we induced Cre-*loxP* recombination on 8-day-old thalli, during the second plastochron. Sectors that were several cells wide emerged along the midrib during the third plastochron. These line-shaped sectors were later separated from the apical notches (Fig. 6a), and were only present on the dorsal surface (Fig. 6b), suggesting a dorsal merophyte origin. Such sectors also overlapped with gemma cups (Fig. 6c), suggesting a minor contribution of dorsal merophytes to gemma cup development. Taken together, these observations suggest that gemma cups arise from dorsal merophytes and dorsal derivatives of lateral merophytes (Fig. 7).

Discussion

Clonal analysis has considerably contributed to understanding plant development. Methodologies for clonal analysis widely vary, involving ploidy changes induced by colchicine (Satina et al. 1940), activation of *GUS* transgene expression by random or heat-shock-mediated removal of transposons (Dolan et al. 1994; Kidner et al. 2000; Scheres et al. 1994), inactivation of *GUS* transgene expression by heat-shock-mediated Cre-*loxP* excision (Saulsberry et al. 2002), pale green pigmentation or chloroplast biogenesis defects induced by X-ray irradiation (Harrison et al. 2007; Harrison et al. 2009), utilisation of variegated species (Sanders et al. 2011), and so on. This paper reports a new clonal analysis method for *M. polymorpha*, which we anticipate will contribute to understanding patterns of *M. polymorpha* development. For the purpose of lineage visualisation, our method could be further improved by incorporating a multi-colour system such as Brainbow (Livet et al. 2007) and Brother of Brainbow (Wachsman et al. 2011). Clonal analysis could be utilised to analyse gene functions in the context of

specific cell lineages or positions (Heidstra et al. 2004; Sieburth et al. 1998). By taking advantage of live imaging, our method could be widely applied to conditional genetic analyses using various molecular tools established in *M. polymorpha* (Ishizaki et al. 2015; Kopischke et al. 2017; Mano et al. 2018; Nishihama et al. 2016; Sugano et al. 2018).

Here, Cre induction in mature gemmae and subsequent growth occasionally generated sectors that bisected thalli and were maintained through bifurcation events, and such sectors are likely to arise by Cre induction in lateral merophytes or apical cells. Some air pores on such sectors were divided into fluorescence-positive and -negative regions (Fig. 4). Air chambers are known to arise from initial apertures that are formed in the limited area around an apical cell, and aperture-surrounding cells later develop into air pores (Apostolakos et al. 1982). Our analysis demonstrated that air pores could emerge across merophyte boundaries as well as within single merophytes, suggesting that air pore formation does not depend on cell lineages but on local cues around the notch (Fig. 7). We further found that most air-chamber sectors traversed air pores, suggesting that cells that constitute air chambers are derived from initial aperture-surrounding cells, which is consistent with the conclusions by Apostolakos et al. (1982).

Gemma cups were also divided by thallus-bisecting sectors (Fig. 5), suggesting that no single initial cell gives rise to gemma cups and that gemma cup development is controlled by local cues. Our observations that the boundary of thallus-bisecting sectors represents the border of lateral merophytes and that dorsal merophyte sectors are incorporated into gemma cups (Fig. 6) demonstrate that gemma cups derive from both dorsal and lateral merophytes (Fig. 7). Though the mechanisms that allow lateral merophytes to contribute differentially to gemma cup formation (Fig. 5d) are unclear, the degree of lateral proliferation of lateral and dorsal merophyte cells may vary depending on the plastochron, or due to plasticity. Recently, an R2R3-type MYB transcription factor, GEMMA CUP-ASSOCIATED MYB1 (GCAM1), was reported to play an essential role in gemma cup formation (Yasui et al. 2019). *GCAM1* is expressed in the notch and the

gemma cup floor and is proposed to maintain dorsal cells near the apical cell in undifferentiated status for gemma cup formation (Yasui et al. 2019). Clarification of the early expression pattern of *GCAMI* in relation to dorsal and lateral merophytes could provide clues to the mechanism of gemma cup formation.

In angiosperms, tissue and organ differentiation is based on cell fates that are determined by positional signals (Kidner et al. 2000; Reinhardt et al. 2005; Scheres 2001; Scheres et al. 1994; Sussex 1951, 1954; van den Berg et al. 1995). Flow of the phytohormone auxin specifies the site of cell differentiation for organ formation, such as leaves and lateral roots. Cell lineages also contribute to the formation of tissue layers, such as the tunica and corpus layers of the shoot apical meristem and the radial layers of root tissues, but their identities are often specified by cell-cell communications with other lineage cells. In *M. polymorpha*, auxin signaling via transcriptional regulation plays critical roles in the development of gemmae through regulation of cell division patterns (Kato et al. 2017). The intermerophyte pattern of organogenesis revealed in this study suggests that similar local signal-mediated cell-fate determination operates in *M. polymorpha*. In contrast, lateral organs in leafy bryophytes arise from merophyte cell lineages with clear histological boundaries (Crandall-Stotler 1980; Harrison et al. 2009; Parihar 1967; Ruhland 1924). Changes in the cell fate determination system may have led to the morphological diversification of complex thalloid liverworts.

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Figures and Figure legends

Fig. 1 Notch bifurcations and dorsal structures of the thallus of *M. polymorpha*.

a Scheme of bifurcating thalli in the n th plastochron stages with exponentially increasing apical notches. Arrowheads indicate apical notches. **b** A 21-day-old *M. polymorpha* thallus in the 4th plastochron. Arrows and Arrowheads indicate gemma cups and apical notches, respectively. Scale bar = 5 mm. **c** Scanning electron microscope (SEM) image of air chambers with one air pore each. Dotted lines and asterisks indicate border of air chambers and air pores, respectively. Scale bar = 200 μ m. **d** SEM image of a gemma cup with serrated edges. Scale bar = 500 μ m. **e** Surface view of initial apertures (grey) which develop into an air pore (Redrawn from Apostolakos et al. (1982)). **f** Vertical longitudinal view of the apical cell and its derivatives. An asterisk, grey area, arrows indicate an apical

cell, gemma cup initial region, young air chambers, respectively (Redrawn from Barnes and Land (1908)). It should be noted that Barnes and Land proposed that initiation of air chambers occurs internally at the intersection of daughter cells from a single mother cell (Barnes and Land 1907), and that the present picture is drawn as such.

Fig. 2 Clonal analysis system in *M. polymorpha*.

a Schematic diagram of the construct used in clonal analysis. In the standard state (top), *tdTomato-NLS* is not transcribed due to an upstream terminator. Heat shock (HS) and dexamethasone (DEX) treatments induce Cre-*loxP*-mediated excision of the *loxP*-

flanked region (bottom), which in turn constitutively switches on *tdTomato-NLS*. *T*:
NOS terminator. **b** Reciprocal formation of GUS and tdTomato sectors. This plant was
treated with HS and DEX in mature gemmae and grown for 14 days. Grey dotted lines
on the left panel indicates approximate position of tdTomato-positive sectors. A white
dotted line on the right panel indicate an outline of the thallus. Bars = 5 mm. **c**
Efficiency of Cre-*loxP* recombination. Mature gemmae of the transgenic line were
treated with or without DEX, and then heat-shocked for 0, 20, 40, or 80 min in a 37 °C
incubator. One day after the induction, the plants showed fluorescence in a HS- and
DEX-dependent manner. Scale bars = 500 µm.

Fig. 3 Sectors that bisect thallus lobes

a Top (upper row) and side (lower row) view of a sector bisecting a thallus lobe in the third plastochron of a 13-day-old plant. Dotted lines indicate the approximate position of the side-view section (basal side observed). Arrowheads indicate apical notches. Scale bars = 500 μ m. **b** A sector bisecting a thallus lobe from the base to an apical notch in the fourth plastochron of a 23-day-old plant. Dotted lines indicate midribs. Arrows and arrowheads indicate gemma cups and apical notches, respectively. Scale bar = 5 mm.

Fig. 4 Division of air pores and air chambers by thallus-bisecting sectors

a A tdTomato-positive sector on dorsal surface of a thallus. Dotted lines in the merged picture indicate the edges of air chambers. Scale bars = 500 μ m. **b** Ratio of relative position of sectors to an air pores (see graph legends and schematic illustrations). $n = 192$

air pores from 10 merophyte sectors. **c, d** A notch-adjacent sector in the second plastochron of a 6-day-old plant (**c**) and its magnified view around the notch within the white dotted rectangle (**d**). As a plasma membrane (PM) marker, mTurquoise2-tagged MpSYP13B (Kanazawa et al. 2016) was co-expressed under the regulation of its native promoter. Arrowheads indicate apical notches. White and yellow arrows indicate air pores formed within and across a sectorized region, respectively. Scale bars = 100 μ m.

Fig. 5 Division of gemma cups by thallus-bisecting sectors

a Correlation between gemma cup positions and plastochron numbers. Circles and differently coloured lines indicate gemma cups and the growth phase of each plastochron, respectively. Gemma cups were never formed during the 1st plastochron under the growth conditions we used. **b** A gemma cup bisected by a merophyte sector. A white dotted line in the merged picture indicates an outline of the gemma cup. Scale bars = 500 μ m. **c** Vertical transverse section of a gemma cup on a merophyte sector. Scale bars = 500 μ m. **d** Dot- and box-plots for the ratio of tdTomato-positive serration edges in a gemma cup formed on a thallus-bisecting sector. The dots are individual values. The bottom and top of each box represent the first and third quartiles, respectively. The band inside the box is the median.

Fig. 6 Division of gemma cups by dorsal merophyte sectors

a–c Top (**a**) and side (**b** and **c**) views of a dorsal line-shaped sector along the midrib that includes a part of gemma cup in the third plastochron of a 23-day-old plant. Scale bars = 500 μm . White dotted lines in **a** indicate the approximate positions of the side-view sections in **b** and **c** (basal sides observed). Arrowheads in **a** indicate apical notches.

Fig. 7 Models of dorsal organ formation in relation to merophytes
Red line and circle in **a** indicate the positions of schematic illustrations for a gemma cup part (vertical transverse section, **b**) and the notch (front view, **c**). Cells in red, yellow, purple, and different levels of green colors in **b** and **c** indicate an apical cell, dorsal merophytes, ventral merophytes, and lateral merophytes, respectively.