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Phytochrome-mediated regulation of cell division and growth during regeneration and sporeling development in the liverwort *Marchantia polymorpha*

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Abstract

Light regulates various aspects of development throughout the life cycle of sessile land plants. Photoreceptors, such as the red (R) and far-red (FR) light receptors phytochromes, play pivotal roles in modulating developmental programs. Reflecting the high developmental plasticity, plants can regenerate tissues, organs, and whole bodies from varieties of cells. Among land plants, bryophytes exhibit extraordinary competency of regeneration under hormone-free conditions. As an environmental factor, light plays critical roles in regeneration of bryophytes. However, how light regulates regeneration remains unknown. Here we show that using the liverwort *Marchantia polymorpha*, which contains a single phytochrome gene, the phytochrome regulates re-entry into the cell cycle and cell shape in newly regenerating tissues. Our morphological and cytological observations revealed that S-phase entry of G_1-arrested epidermal cells around the midrib on the ventral surface of thallus explants was greatly retarded in the dark or under phytochrome-inactive R/FR cycle irradiation conditions, where, nevertheless, small, laterally narrow regenerants were eventually formed. Thus, consistent with earlier description published over a century ago, light is not essential for but exerts profound effects on regeneration in *M. polymorpha*. Ventral cells in regenerants grown under R/FR cycle conditions were longer and narrower than those under R cycle. Expression of a constitutively active mutant of *M. polymorpha* phytochrome allowed regeneration of well grown, widely expanded thalli even in the dark when sugar was supplied, further demonstrating that the phytochrome signal promotes cell proliferation, which is rate-limited by sucrose availability. Similar effects of R and FR irradiation on cell division and elongation were observed in sporelings as well. Thus, besides activation of photosynthesis, major roles of R in regeneration of *M. polymorpha* are to facilitate proliferation of rounder cells through the phytochrome by mechanisms that are likely to operate in the sporeling.

Keywords

Cell division cycle, cell shape, dedifferentiation, phytochrome, regeneration, sugar signaling

Introduction

Plants, photoautotrophic organisms, seek light as a means of survival. Aquatic green algae with flagella are able to move to suitable environment. Sessile land plants, however, cannot adopt this strategy. Consequently, they created ingenious mechanisms to adapt to various light conditions and adjust their growth and morphology. For instance, under low-light conditions, seed plants elongate various parts, including stems and petioles, and facilitate the escape from the unfavorable environment, which is known as shade avoidance response (Casal 2013). By contrast, when sufficient light is available, plants operate the program called photomorphogenesis for the formation of photosynthetic organs and their lateral expansion to maximize light absorption (Kami et al. 2010).

These light responses are mediated by several pathways. First, light is perceived by chloroplasts and used for photosynthesis, which generates chemical energy and various signals, such as sugars and retrograde signals. Light is also perceived by several kinds of photoreceptors: the red-light (R)/far-red-light (FR) receptor phytochrome; the blue-light receptors cryptochrome, phototropin, and the ZEITLUPE/FLAVIN BINDING, KELCH REPEAT, F-BOX1 (FKF1)/LOV KELCH PROTEIN2 family; and the UV-B receptor UV RESISTANCE LOCUS8 (Christie 2007; Fraikin et al. 2013; Franklin and Quail 2010; Ito et al. 2012; Kami et al. 2010; Liu et al. 2011). Among these, phytochromes have a special feature that their activities can be quickly converted between the active, FR-absorbing Pfr form and the inactive, R-absorbing Pr form by R and FR light, respectively (Franklin and Quail 2010; Kami et al. 2010). This photoconversion can allow recognition of environment differences between light and shade and according modulation of gene expression patterns (Casal 2013).

Light responses regulated by phytochromes are diverse: some include seed germination, de-etiolation, photoperiodicity, and transition from vegetative to reproductive phase in seed plants. At the cellular level, phytochromes are involved in regulation of cell proliferation and growth. In Arabidopsis etiolated seedlings, light-activated cell proliferation leading to rosette leaf formation is mediated at least in part by phytochromes (López-Juez et al. 2008). Also in basal land plants, including bryophytes and pteridophytes, the phytochrome system has been implicated in controlling a number of processes, such as germination of spores (reviewed in Nishihama and Kohchi 2013; Wada and Kadota 1989) and cell cycle, elongation, branching, and phototropism of protonemata (Brücker et

al. 2005; Hartmann et al. 1983; Kadota et al. 1982; Mittmann et al. 2004; Wada 1985; Wada et al. 1984) in a variety of species.

Bryophytes, including liverworts, mosses, and hornworts, have been known for their extraordinary efficiency of regeneration since the 18th century bofore the discovery of phytohormones. Necker (1774) described regeneration of liverworts in his book. Over a century later, Vöchting (1885) reported that using thalli and gemmae of the liverworts *Lunularia vulgaris* (=*cruciata*) and *Marchantia polymorpha*, regeneration requires removal of the apex, a growth point that contains an apical cell and surrounding meristematic cells, and that regenerants are formed from the apical and ventral sides of apex-removed explants. Then, it was reported that almost all cell types in the gametophyte generation could regenerate in liverworts (Cavers 1903; Kreh 1909; Schostakowitsch 1894). Furthermore, Heald (1898b) also referred to light effect on regeneration of mosses and noted light dependency for many species. Absolute requirement for light in regeneration from protoplasts was shown in *Physcomitrella patens* (Jenkins and Cove 1983), and the phytochrome system was implicated in facilitation of regeneration for *Mnium affine* (Giles and von Maltzahn 1967). Regeneration in liverworts is also greatly facilitated by light. Cavers (1903) reported feeble growth of regenerants in the dark in several liverwort species, including *M. polymorpha*.

One of the mechanisms for regeneration in both plants and animals involves dedifferentiation of differentiated cells into a totipotent/pluripotent status, which is followed by cell proliferation and redifferentiation. At least in some mosses, leaf cells directly (that is without cell division) revert back to the protonema stage upon excision (Giles 1971; Ishikawa et al. 2011; Prigge and Bezanilla 2010). Likewise, regeneration of liverworts has been suggested to recapitulate the development through sporelings (Kreh 1909). Protonemata and sporelings are common in terms of their developmental stages that immediately follow spore germination. Recent study revealed that activation of cyclin-dependent protein kinase A, a core cell-cycle regulator in plants, is essential for protonema regeneration in *P. patens* (Ishikawa et al. 2011). However, how light regulates regeneration has remain elusive for any bryophytes.

To address this question, we focused on the liverwort *M. polymorpha*, which has a long history of regeneration research as mentioned above. Recent technical advances, such as high-efficiency transformation with sporelings and regenerating thalli (Ishizaki et al. 2008; Kubota et al. 2013),

homologous recombination-based gene targeting (Ishizaki et al. 2013), and CRISPR/Cas-mediated genome editing (Sugano et al. 2014) have enabled us to use *M. polymorpha* for molecular genetic studies. In addition, genome and transcriptome projects are under way by the Community Sequencing Program in U.S. Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/). *In silico* searches revealed the presence of all the major photoreceptors with low genetic redundancy in *M. polymorpha*, and the phototropin and FKF1 orthologs were shown to be involved in chloroplast photorelocation movement and photoperiodic growth-phase transition, respectively (Komatsu et al. 2014; Kubota et al. 2014). In this study, we examined effects of phytochrome activity and sugar availability on cell proliferation and growth after thallus excision and spore germination to gain insights into the roles of light signaling mediated by the phytochrome and photosynthesis-derived sugar in regeneration in *M. polymorpha*.

Materials and methods

Plant material and growth media

Female and male *M. polymorpha* accessions, Takaragaike-2 (Tak-2) and Tak-1, respectively (Ishizaki et al. 2008), were cultured aseptically on half-strength Gamborg's B5 medium (Gamborg et al. 1968) containing 1 % agar. Spores were obtained by crossing Tak-2 and Tak-1. Generation of transgenic plants with *M. polymorpha PHYTOCHROME* (*MpPHY*) will be described elsewhere.

Light conditions

Light conditions used in this study are as follows. Continuous white: 50–60 μmol photons m^{-2} s^{-1} with cold cathode fluorescent lamp (OPT-40C-N-L, Optrom); R cycle: repetition of a 3-min cycle consisting of R irradiation (35 μmol photons m^{-2} s^{-1}; MIL-R18, SANYO Electric) for 40 s and darkness for 140 s; R/FR cycle: R irradiation (35 μmol photons m^{-2} s^{-1}) for 40 s, FR (40 μmol photons m^{-2} s^{-1}; MIL-IF18, SANYO Electric) for 40 s, and darkness for 100 s.

Regeneration assay

Gemmae were plated on half-strength Gamborg's B5 plates and incubated at 22 ˚C for 10–14 days under continuous white light. Thalli were cut off with a scalpel on a plastic petri dish. Explants were

transferred onto half-strength Gamborg's B5 plates with or without 1 % sucrose and incubated at 22 ˚C under various light conditions. Micrographs were captured using SZX16 stereoscope (Olympus) equipped with DP20 cooled charge-coupled device (CCD) camera (Olympus).

Spore growth assay

Surface-sterilized spores were plated on half-strength Gamborg's B5 plates that contained 0.01 % glucose and 1.6 % gellan gum and incubated at 22 ˚C for 10 days under R or R/FR cycles. Micrographs were taken through Axiophot microscope (Zeiss) equipped with DP72 cooled CCD camera (Olympus).

Flow cytometry

Flow cytometry was performed essentially as previously described (Galbraith 2009). All procedures were performed on ice. About 50 mg of basal fragments of *M. polymorpha* thalli and Arabidopsis rosette leaf were chopped with a razor blade for ~2 min in the presence of 1 ml of Galbraith's buffer (45 mM magnesium chloride, 30 mM sodium citrate, 20 mM 4-morpholinepropane sulfonate, 0.1 % Triton X-100; Galbraith et al. 1983). Homogenates were filtered through 40µm Cell Strainer (BD Biosciences), and 2.5 µl of 10 mg ml^{-1} RNase A (Sigma-Aldrich) was added to 500 µl of the cleared homogenates, followed by incubation on ice for 15 min. Propidium iodide (nacalai tesque) was added to a final concentration of 50 µg ml^{-1}. Samples were incubated on ice for at least 30 min before applying to a flow cytometer (BD Accuri C6; BD Biosciences).

Visualization of S-phase cells

S-phase cells were visualized using Click-iT EdU Imaging Kits (Life Technologies) basically according to the manufacturer's instruction. Explants of Tak-1 thalli grown for 10 days from gemmae were incubated on half-strength Gamborg's B5 plates supplemented with or without 1 % sucrose under various light conditions. At various time points, explants were transferred to a tube containing the identical medium with 10 µM 5-ethynyl-2'-deoxyuridine (EdU) and incubated for 4 h at the same light conditions. Incorporation of EdU was terminated by fixing the explants with 3.7 % formaldehyde solution in phosphate-buffered saline (PBS) for 20 min. The explants were washed twice with PBS,

permeabilized with 0.5 % Triton X-100 in PBS for 20 min, and washed twice with PBS containing 3 % bovine serum albumin (PBS+BSA). EdU incorporated into DNA was stained by incubation in the dark with Alexa Fluor 555-azide-containing Click-iT reaction cocktail for 30 min. After washing once with PBS, DNA was stained with 1 μg ml^{-1} DAPI in PBS for 30 min in the dark and washed twice with PBS. Z-series of 10–20 fluorescent images with 2-μm steps were captured using a confocal laser scanning microscope, FV1000 (Olympus; 20× UPLAPO objective lens; N.A. = 0.70). DAPI was excited by 405-nm laser and detected with 425–475 nm window; Alexa Fluor 555 was excited by 546-nm laser and detected with 555–625 nm window. Z-projection images were created using the ImageJ software (Schneider et al. 2012).

Observation by SEM

SEM was performed using Miniscope TM-3000 (Hitachi) according to the manufacture's instruction. Briefly, plants were put on a carbon tape adhered to the sample holder and rapidly frozen in liquid nitrogen for 40 s. The holder was immediately placed in the sample chamber, which was vacuumed before observation.

Measurement of cell morphology

All the measurements were performed by ImageJ using SEM or light microscopic images. For measurement of cell length with SEM images, maximal-length straight lines manually drawn on individual cells were measured. For cell surface area, cell boundaries were traced manually with the polygon selection tool and measured. For aspect ratio, the same polygon selections were converted to the best fitting ellipses, and the ratios of major axis to minor one were determined. For measurement of cell length in sporelings with light microscopic images, segmented lines manually drawn to connect the centers of two adjacent cell walls were measured.

Results

Cellular processes for regeneration from thallus explants in *M. polymorpha*

Our previous study suggested that the visible outgrowth of regenerating plants after excision of *M. polymorpha* thallus was observed within 5 days (Kubota et al. 2013). As previously reported

(Vöchting 1885), regeneration occurred predominantly from the apical end of basal explants (Supplemental Fig. S1). To understand the course of cellular events during regeneration in more detail, we first performed time-lapse observation of basal explants in 1-h intervals under continuous white light (Fig. 1; Supplemental Movie 1). The first observed morphological change was rhizoid elongation, which initiated immediately after excision and became conspicuous by 6 h. No detectable change was observed on the excision surface until around 60 h, when a bulge of tissue was formed near the midrib. Around 96 h, outgrowth of thallus-like regenerating plants was observed. These results suggest that cell division was activated within 60 h after excision at the latest.

Observation by SEM of explants bearing readily recognizable regenerants revealed that they had emerged from the ventral side of the thallus, while there was no visible morphological change on the dorsal surface (Fig. 2h; note that there was no regeneration from the dorsal surface nor the parenchyma tissue). We therefore examined earlier morphological changes of the cells on the ventral side after excision (see Supplemental Fig. S2a for the excision pattern; Supplemental Fig. S3a, b for the architecture of the ventral side of a thallus). Epidermal cells on the midrib, which could be recognized by the presence of smooth rhizoids, were smaller than those in the thallus blade (Fig. 2a). There was no noticeable difference in cellular morphology up to 24 h after excision (Fig. 2a, b). By 36 h, most of the ventral epidermal cells on the midrib and some of those in the thallus blade near the midrib displayed one shallow groove in their middle, which appeared to be contours of newly formed cell plates (Fig. 2c), therefore suggesting the occurrence of first cell divisions. During the next 12 h, one or two more rounds of cell divisions appeared to occur in both midrib and thallus blade regions (Fig. 2d, e). By this time, grooves that were derived from the original boundaries of epidermal cells became deeper, probably due to a slight increase in volume of the daughter cells. Further cell divisions created bulges of cells by 72 h (Fig. 2f). By this time, almost all the ventral epidermal cells appeared to have re-entered the cell cycle (Supplemental Fig. S3c). At 96 h, various types of differentiated cells were clearly observed, including air-pore cells, slime papillae, and rhizoids (Fig. 2g–i), suggesting that the developmental program began to operate some time between 72 h and 96 h.

Next, timing of re-entry into the cell cycle was examined. To know the cell cycle stage of the cells in the basal part of the thallus, we conducted flow cytometric analysis, using Arabidopsis rosette leaf, which contains various degrees of endoreduplicated cells, as a control for nuclear DNA content

(Galbraith 2009). Estimated haploid genome sizes of Arabidopsis and *M. polymorpha* are ~135 Mb (http://arabidopsis.org/portals/genAnnotation/gene_structural_annotation/agicomplete.jsp) and ~280 Mb (Okada et al. 2000), respectively. *M. polymorpha* sample showed only one peak with the fluorescence intensity comparable to the 2C-value peak of Arabidopsis leaf sample (Fig. 3), suggesting that the thallus cells contain 1C nuclear DNA content and, therefore, are arrested in G_1 phase. Incorporation assays of EdU, a thymidine analog that allows identification of S-phase nuclei, revealed that although there was no vigorous EdU incorporation in the first 16 h after excision (see Supplemental Fig. S2b for the excision pattern), sudden increase in the number of EdU-incorporated nuclei was observed during the next 12 h (Fig. 4a), indicating that the cells entered S phase between 16 and 28 h after excision. We also examined the influence of sucrose to the timing of S-phase entry, because sugars have been reported to promote cell cycle progression (Riou-Khamlichi et al. 2000; Xiong et al. 2013). However, essentially the same patterns of EdU incorporation were observed regardless of the presence or absence of 1 % sucrose (cf. Fig. 4e vs. Fig. 4a), suggesting that sugar had no effect on the timing of S-phase entry under the continuous white light conditions used in this study.

Effects of light on regeneration

In mosses, regeneration is known to be light-dependent and suggested to be phytochrome-regulated (Giles and von Maltzahn 1967; Jenkins and Cove 1983). We therefore examined light dependency and effect of R and FR irradiation on the regeneration of *M. polymorpha*. In the absence of light, explants (see Supplemental Fig. S2b for the excision pattern) that had been incubated on sugar-free medium for 2 weeks produced one or two narrow regenerants per explant, but they failed both to grow out vigorously and expand laterally (Fig. 5a, Dark). Addition of 1 % sucrose to the medium appeared to promote the growth of regenerants and their rhizoids but did not allow development into laterally expanded thalli (Fig. 5b, Dark). These results suggest that light is not essential for the initiation of regeneration. Nonetheless, comparison with regenerants incubated under white light (Fig. 5a, b, cW) indicates that light exerts strong stimulating effects on the growth of regenerants and their development into thalli, which cannot be recapitulated simply by supplying sugar in the dark.

To examine the impact of irradiation of R and FR to regeneration, explants were incubated under the following two light conditions: phytochrome-active R cycle, intermittent irradiation cycles

consisting of 40-s of R and 140-s of darkness; phytochrome-inactive R/FR cycle, intermittent irradiation cycles consisting of 40-s of R, 40-s of FR, and 100-s of darkness (Supplemental Fig. S4). Regardless of sucrose, R cycle irradiation promoted lateral growth of regenerants and development into thalli, although the sizes of regenerants were smaller in the absence of sucrose than in its presence (Fig. 5a, b, R cycle), which would be due to a non-maximal photosynthesis rate under the light conditions used. Under R/FR cycle in the absence of sucrose, the explants regenerated a few narrow structures (Fig. 5a, R/FR cycle), which were morphologically similar to those formed in the dark with sucrose, including rhizoid outgrowth (Fig. 5b, Dark). These results suggest that the growth of regenerants is regulated positively by the phytochrome. Unexpectedly, in the presence of 1 % sucrose, regenerants formed under R/FR cycle were as nearly large as those under R cycle (Fig. 5b). This could be due to a residual activity of phytochrome under the R/FR cycle or some other signaling events triggered by light, which could have been sufficient for the growth of regenerants when sucrose, most likely as chemical energy, was exogenously supplied.

EdU incorporation analysis revealed that, in the absence of exogenous sugar, R cycle irradiation allowed S-phase entry by 28 h after excision (Fig. 4c), the same timing as the continuous white conditions, whereas dark or R/FR cycle conditions did not promote S-phase entry at least until 40 h (Fig. 4b, d). In the presence of 1 % sucrose, S-phase entry was detected by 28 h under both R and R/FR cycle conditions and by 40 h in the dark. SEM analysis showed the occurrence of cell divisions by 96 h under all the conditions tested (Supplemental Fig. S5). These data suggest that the timing of cell cycle re-entry depends on the active state of phytochrome and that excess sugar can override the phytochrome regulation to promote S-phase entry.

Facilitating growth and development of regenerants by active phytochrome

To explore the role of the phytochrome more directly, we examined effects of overexpression of a putatively dominant-active phytochrome mutant on regeneration under various light conditions. We modified the phytochrome gene in *M. polymorpha* (*MpPHY*) to introduce an amino-acid substitution of the conserved Tyr241 residue to His ($MpPHY^{Y241H}$; Supplemental Fig. S6). The same substitution of Tyr276 to His in Arabidopsis phyB was shown to confer photoinsensitivity and therefore constitutive functionality (Hu et al. 2009; Su and Lagarias 2007). Transgenic *M. polymorpha* lines

($_{pro}$*EF:MpPHY*^{Y241H}) that overexpressed this mutant under the control of *MpEF1α* promoter (Althoff et al. 2014) were subjected to the regeneration assay as above.

In the absence of exogenous sugar, these plants formed fully developed, regenerated thalli under continuous white light, whereas they showed minimal activity of regeneration in the dark (Fig. 5c, cW, Dark), as observed for wild-type plants (Fig. 5a). Thus, modulation of the phytochrome activity does not affect the regeneration process under these light and growth conditions. However, although no big difference on regeneration was observed under the R cycle between $_{pro}$*EF:MpPHY*^{Y241H} and wild-type explants, the former lines regenerated laterally expanded thalli even under the R/FR cycle (Fig. 5c). These results support the assumption that the phytochrome with Y241H substitution is constitutively active and suggest the role of the phytochrome in lateral growth of regenerants.

In the presence of sucrose, $_{pro}$*EF:MpPHY*^{Y241H} explants produced well developed, much larger thalli than wild type not only under the R cycle but also under the R/FR cycle and even in the dark (Fig. 5d). The size and morphology of the regenerants were similar under all these conditions, implying that the signaling activity of the phytochrome was saturated in these lines. Taken together, these results suggest that phytochrome signaling can drive both vigorous growth and lateral expansion of regenerants, when sugar is exogenously supplied.

Regulation of cell shape by the phytochrome

To understand the basis of developmental regulation of regenerants by the phytochrome, regenerants grown under R or R/FR cycle for 14 days were observed by SEM. Laterally expanding thallus-like regenerants that had been formed under R cycle showed dorsoventrality, whose ventral side contained rhizoids and scales in the middle region (Fig. 6a–c; Supplemental Fig. S7a, b). Upright and narrow regenerants that had been formed under R/FR cycle also retained dorsoventrality with the surface distal to the explant being the ventral side, as evidenced by rhizoid and scale formation on that side (Fig. 6d–f; Supplemental Fig. S7c, d) and air chamber formation, albeit rarely, on the other side (Supplemental Fig. S7d). These results suggest that the formation of narrow structures is not due to a loss of dorsoventrality.

Comparison of the morphology of cells in the ventral epidermis of the R-cycle regenerant versus that in its corresponding region of the R/FR-cycle regenerant revealed no significant difference in cell

surface area (Fig. 6g). However, cells on the R/FR-cycle regenerant were significantly longer in length than those on R-cycle regenerant (Fig. 6h). More drastically, aspect ratio (major to minor axis lengths of fitted ellipses) was much larger in the R/FR-cycle regenerant (Fig. 6i). These data demonstrate that R and FR affect cell shape at least in certain cell type with R promoting formation of rounded cells.

Roles of the phytochrome in cell division and elongation in sporelings

To examine the effect of phytochrome signaling on cell division and growth in different cell types, spores were cultured under R or R/FR cycle. During 10 days of culture under the R cycle, germinated spores underwent multiple rounds of cell divisions and branching, while under the R/FR cycle conditions, cells did not divide much and, instead, elongated tremendously (Fig. 7a). Counting the number of cells and branches per sporeling revealed that the R cycle was more effective to facilitate cell division and branching than the R/FR cycle (Fig. 7b, c). In addition, lengths of individual cells were much longer under the R/FR cycle than the R cycle (Fig. 7d). These results suggest that in sporelings, the Pfr form of the phytochrome plays a role in promoting cell division and branching, while cell elongation is a preferred cellular process when the phytochrome is inactive.

Discussion

Cellular events and morphological changes during regeneration

Our analyses revealed a series of cellular events and morphological changes that occur during regeneration from excised thalli of *M. polymorpha*. The first obvious event was rhizoid growth near the midrib, which initiated immediately after excision and ceased when a bulge of tissue was formed (Fig. 1; Supplemental Movie 1). It remains to be clarified whether this is a response to excision or just continuation of developmentally regulated growth of rhizoid at the excised site. At later time points, rhizoid formation was reinitiated from regenerants (Fig. 2h). Interestingly, incubation in the dark without sugar resulted in no conspicuous rhizoid formation, whereas that with sucrose allowed it (Fig. 5). Moreover, regenerants grown under R or R/FR cycle in the absence of sugar formed rhizoids. These results suggest that photosynthetically produced sugar plays a critical role in rhizoid formation. It would be intriguing to examine which step sugar regulates, differentiation of the rhizoid initial or elongation of single-cell rhizoids from initials.

The next observed event was activation of the cell cycle. Predominant accumulation of 1C DNA-containing cells in the basal part of thalli (Fig. 3), occurrence of nuclear DNA replication around 24 h after excision (Fig. 4a, e), and appearance of dividing cells by 36 h (Fig. 2c) suggest that thallus cells quiescent in G_1 phase entered into S phase upon excision. This is in contrast to the case of regeneration from leaf cells in the moss *P. patens*, in which leaf cells are arrested in late S phase and re-enter the cell cycle by peforming some kind of DNA synthesis (Ishikawa and Hasebe 2015; Ishikawa et al. 2011).

These cell cycle events took place first along the midrib on the ventral surface in the apical end. Correlated with this, regenerants were formed from the ventral side of the apical end, and no noticeable change was observed on the dorsal surface (Fig. 2h). These observations are consistent with the earlier study by Vöchting (1885) describing that two Marchantiopsida liverworts, *L. vulgaris* and *M. polymorpha*, almost always produce regenerants at the apical end of the explant, and those by Schostakowitsch (1894) and Goebel (1907) demonstrating that thalloid liverworts favor regeneration from the ventral side near the midrib. In the case of Arabidopsis regeneration assisted by phytohormones, callus formation occurs from a specific cell type, pericycle cells in root or pericycle-equivalent cells in other organs, acting as adult stem cells that 'differentiate' into root meristem-like tissue (Sugimoto et al. 2010). Ventral epidermal cells on the midrib in *M. polymorpha*, which are similar in size to cells in the meristematic region (cf. Fig. 2a, b vs. Supplemental Fig. S3b), might retain adult stem cell-like properties. Our data, however, demonstrated that larger and seemingly completely quiescent ventral epidermal cells in the thallus blade re-entered the cell cycle in the same timing as those on the midrib (Fig. 2c–e) and that eventually, almost all ventral epidermal cells underwent cell division (Supplemental Fig. S3c), suggesting that dedifferentiation of cells that have been differentiated at least to some extent does occur during regeneration in *M. polymorpha*. Further detailed analysis with cell type-specific markers should clarify differentiation status of ventral epidermal cells on the midrib.

Regeneration in mosses is known to involve direct conversion of differentiated single cells into elongated protonemata (Giles 1971; Heald 1898b; Prigge and Bezanilla 2010) even before cell division (Ishikawa et al. 2011). It has been reported that most liverwort species do not exhibit this elongation feature and recapitulate the development through sporelings (Kreh 1909; Rickett 1920;

Schostakowitsch 1894), although Kaul et al. (1962) later clearly demonstrated that in *M. nepalensis*, the regeneration goes through a short protonemal phase. In some liverworts, such as *Preissia commutata*, filamentous growth could be induced when sporelings or prothalli are transferred to weak light conditions (Goebel 1898; Hansel 1876; Kreh 1909; Schostakowitsch 1894). Our SEM analyses, however, demonstrated that no protonema-like elongated cell was generated on the ventral surface of 96-h-old *M. polymorpha* explants even in the dark or under R or R/FR cycle before the appearance of dividing cells or the formation of cell bulge, regardless of sugar application (Supplemental Fig. S5). Sporelings of *M. polymorpha* did elongate in response to the same R/FR conditions (Fig. 7). Thus, the cell differentiation status of regenerating cells might not be identical to that of sporelings in *M. polymorpha*.

Nonetheless, developmental processes of regenerants are indeed analogous to those of thalli from spores. A germinated spore repeats rounds of cell divisions. During this sporeling stage, an apical cell is generated at the tip, and development into a thallus takes place. It seemed that, at the initial stage of regeneration, a unit of cells derived from each ventral epidermal cell (Fig. 2d, e) corresponded to a single sporeling. This idea is supported by the formation of ball-shaped tissues or tissue blocks separated each other (Fig. 2f, h) and later formation at various locations of a number of slime papillae (Fig. 2g, i), which, in thalli, are formed only a few per apical cell. The observation that only one or two regenerating thalli were developed from one bulge (Fig. 1) suggests possible existence of a lateral inhibition mechanism that operates among apical cells in neighboring sporeling units. It would be intriguing to understand how apical cells are established during regeneration.

Photoregulation of cell division during regeneration

EdU incorporation analysis performed with sugar-free media revealed substantial delay in the excision-induced entry into S phase in the dark or under R/FR cycle (Fig. 4). No delay under R cycle suggests that the phytochrome plays a critical role in promoting cell cycle re-entry of the quiescent epidermal cells. This regulation also functions in cycling cells, as the frequency of cell division in sporelings was under the regulation of the phytochrome (Fig. 7). Most importantly, overexpression of the constitutively active phytochrome in the dark or under R/FR cycle in the presence of sugar resulted

in quite as large regenerants as those under R cycle (Fig. 5d), which clearly shows that cell division activity can be drastically stimulated by the active phytochrome (Fig. 8).

Phytochrome-dependent activation of cell cycle has been reported for evolutionarily diverged plant species. In the fern *Adiantum capillus-veneris*, the first cell cycle of spores exhibits clear phytochrome dependency (Furuya et al. 1997). In the shoot apical meristem of etiolated Arabidopsis seedlings, quiescent cells can be quickly activated to re-enter the cell cycle by light irradiation in a manner dependent on phytochromes and cryptochromes (López-Juez et al. 2008). During this process, most of the core cell-cycle regulator genes are up-regulated with similar timings (López-Juez et al. 2008). However, molecular mechanisms that link the photoreceptors to cell cycle activation remain to be elucidated in any land plant species.

During regeneration of *M. polymorpha* thalli in the presence of sucrose, S-phase entry was observed in almost the same timing regardless of light conditions, indicating that sucrose also promotes cell cycle re-entry independently of the phytochrome (Fig. 8). Thus, cells undergo dual cell-cycle regulation by the phytochrome and sugar. Effectiveness of sugar in the dark suggests that photosynthesis stimulates cell cycle progression through sugar production. The reason why R/FR cycle did not allow normal S-phase entry on sugar-free medium even with a photosynthesis rate predicted to be identical to that under R cycle could be insufficient sugar production with the strength of R used in this study, a notion supported by formation of much smaller regenerants under R cycle compared with cW. A link between sugar and cell cycle has been reported in angiosperms. In Arabidopsis, sucrose and glucose can induce expression of cyclin D genes (Riou-Khamlichi et al. 2000; Soni et al. 1995), which encode G_1/S cyclins that promote transition from G_1 phase to S phase. More recently, in Arabidopsis roots, glucose was shown to activate a kinase, TARGET OF RAPAMYCIN (TOR), which in turn directly phosphorylates and activates the transcription factor E2 promoter binding factor (E2F) that promotes S-phase gene transcription (Xiong et al. 2013). It would be interesting to investigate whether these cell cycle regulations by sugar commonly operate in land plants.

Photomorphogenesis of regenerants and sporelings

In contrast to the regeneration of laterally expanded thallus structures under R cycle, regenerants formed under R/FR cycle were narrow, although their lengths were quite similar between the two conditions (Figs. 5, 6; Supplemental Fig. S7). The latter regenerants exhibited a rod-like appearance but still retained the dorsoventral pattern of the original thalli, which confirms Vöchting's observation (Vöchting 1885). Our quantitative measurement of cell parameters suggests that the phytochrome regulates cell shape. In ventral epidermal cells of regenerants, when the phytochrome was active, cells tended to expand more non-isotropically, while when inactive, cell elongation along the growth axis was facilitated (Fig. 6). No observed difference in cell-surface area suggests that the degree of cell expansion was comparable. Thus, the phytochrome is likely to be involved in the regulation between directional and non-directional growth of cells (Fig. 8), contributing at least in part to the morphological differences.

In angiosperms, a low R:FR ratio environment induces shade avoidance responses, by which stems and petioles become elongated (Casal 2013; Neff et al. 2000). The length of cells in sporelings was significantly longer under R/FR cycle than R cycle (Fig. 7), indicating that inactivation of the phytochrome leads to elongation of the sporeling cells. These elongation responses in sporelings as well as in thalli could be viewed as shade avoidance responses.

Regeneration and light in bryophytes

Heald (1898b) reported that, in mosses, protonema regeneration is strictly light-dependent in some species but not in others. *P. patens* belongs to the former and absolutely requires light for regeneration from protoplasts (Jenkins and Cove 1983). Heald (1898b) also described the occurrence of regeneration of *M. polymorpha* in the dark. Later, Cavers (1903) reported slow growth of regenerants in the dark for several liverwort species including *M. polymorpha*. Thus, together with these early studies, our results suggest that the initial trigger for regeneration of *M. polymorpha* may not be light. Classic studies by Vöchting (1885) and Dickson (1932) showing that removal of the apex is critical and partial excision is not sufficient for regeneration strongly suggest a plausible hypothesis that a relief from putative inhibition by the apex triggers regeneration and also suggest that wounding itself cannot trigger regeneration (Fig. 8).

As described above, in bryophyte regeneration, differentiated cells can revert back to protonema or sporeling stages. However, it remains to be clarified whether the cells directly revert to those cell types or undergo dedifferentiation to a precursor stage, namely spores, before entering the protonema or sporeling stage. If the latter is the case, light-dependency of regeneration could reflect that of spore germination. In most bryophyte species, spore germination depends on the light (Heald 1898a; Nishihama and Kohchi 2013; Wada and Kadota 1989), and, for many mosses, such as *P. patens*, *Ceratodon purpureus*, and *Dicranum scoparium*, light signal is mediated by the phytochrome (Bauer and Mohr 1959; Cove et al. 1978; Valanne 1966). Interestingly, *M. polymorpha* does not utilize the phytochrome system, but responds to sugar production by photosynthesis for the onset of spore germination, which therefore could be induced by sugar even in the dark (Nakazato et al. 1999). This feature, by using sugar stored in the thallus, might also allow recapitulation of sporeling development from dedifferentiated cells with a spore-like status during regeneration in the dark.

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References

Althoff F, Kopischke S, Zobell O, Ide K, Ishizaki K, Kohchi T, Zachgo S (2014) Comparison of the *MpEF1α* and *CaMV35* promoters for application in *Marchantia polymorpha* overexpression studies. Transgenic Res 23:235-244

Bauer L, Mohr H (1959) Der Nachweis des reversiblen Hellrot-Dunkelrot-Reaktionssystems bei Laubmoosen. Planta 54:68-73

Brücker G, Mittmann F, Hartmann E, Lamparter T (2005) Targeted site-directed mutagenesis of a heme oxygenase locus by gene replacement in the moss *Ceratodon purpureus*. Planta 220:864-874

Casal JJ (2013) Photoreceptor signaling networks in plant responses to shade. Annu Rev Plant Biol 64:403-427

Cavers F (1903) On asexual reproduction and regeneration in Hepaticae. New Phytol 2:121-133

Christie JM (2007) Phototropin blue-light receptors. Annu Rev Plant Biol 58:21-45

Cove DJ, Schild A, Ashton NW, Hartmann E (1978) Genetic and physiological studies of the effect of light on the development of the moss, *Physcomitrella patens*. Photochem Photobiol 27:249-254

Dickson H (1932) Polarity and the production of adventitious growing points in Marchantia polymorpha. Ann Bot 46:683-684

Fraikin GY, Strakhovskaya MG, Rubin AB (2013) Biological photoreceptors of light-dependent regulatory processes. Biochemistry (Mosc) 78:1238-1253

Franklin KA, Quail PH (2010) Phytochrome functions in *Arabidopsis* development. J Exp Bot 61:11-24

Furuya M, Kanno M, Okamoto H, Fukuda S, Wada M (1997) Control of mitosis by phytochrome and a blue-light receptor in fern spores. Plant Physiol 113:677-683

Galbraith DW (2009) Simultaneous flow cytometric quantification of plant nuclear DNA contents over the full range of described angiosperm 2C values. Cytometry A 75:692-698

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220:1049-1051

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151-158

Giles KL (1971) Dedifferentiation and regeneration in bryophytes: A selective review. NZ J Bot 9:689-694

Giles KL, von Maltzahn KE (1967) Interaction of red, far-red, and blue light in cellular regeneration of leaves of *Mnium affine*. Bryologist 70:312-315

Goebel K (1898) Archegoniatenstudien. VIII. Rückschlagsbildungen und Sprossung bei *Metzgeria.* Flora 85:69-74

Goebel K (1907) Archegoniatenstudien. XI. Weitere Untersuchungen über Keimung und Regeneration bei *Riella* und *Sphaerocarpus*. Flora 97:192-215

Hansel V (1876) Über die Keimung der *Preissia commutata* N. v. E. Sitzungsber Kais Akad Wissensch (Wien) 73:89-97

Hartmann E, Klingenberg B, Bauer L (1983) Phytochrome-mediated phototropism in protonemata of the moss *Ceratodon purpureus* Brid. Photochem Photobiol 38:599-603

Heald FdF (1898a) Conditions for the germination of the spores of bryophytes and pteridophytes. Bot Gaz 26:25-45

Heald FdF (1898b) A study of regeneration as exhibited by mosses. Bot Gaz 26:169-210

Hu W, Su YS, Lagarias JC (2009) A light-independent allele of phytochrome B faithfully recapitulates photomorphogenic transcriptional networks. Mol Plant 2:166-182

Ishikawa M, Hasebe M (2015) Cell cycle reentry from the late S phase: implications from stem cell formation in the moss *Physcomitrella patens*. J Plant Res (this issue). doi:10.1007/s10265-015-0713-z

Ishikawa M, Murata T, Sato Y, Nishiyama T, Hiwatashi Y, Imai A, Kimura M, Sugimoto N, Akita A, Oguri Y, Friedman WE, Hasebe M, Kubo M (2011) *Physcomitrella* cyclin-dependent kinase A links cell cycle reactivation to other cellular changes during reprogramming of leaf cells. Plant Cell 23:2924-2938

Ishizaki K, Chiyoda S, Yamato KT, Kohchi T (2008) *Agrobacterium*-mediated transformation of the haploid liverwort *Marchantia polymorpha* L., an emerging model for plant biology. Plant Cell Physiol 49:1084-1091

Ishizaki K, Johzuka-Hisatomi Y, Ishida S, Iida S, Kohchi T (2013) Homologous recombination-mediated gene targeting in the liverwort *Marchantia polymorpha* L. Sci Rep 3:1532

Ito S, Song YH, Imaizumi T (2012) LOV domain-containing F-box proteins: light-dependent protein degradation modules in *Arabidopsis*. Mol Plant 5:573-582

Jenkins GI, Cove DJ (1983) Light requirements for regeneration of protoplasts of the moss *Physcomitrella patens*. Planta 157:39-45

Kadota A, Wada M, Furuya M (1982) Phytochrome-mediated phototropism and different dichroic orientation of Pr and Pfr in protonemata of the fern *Adiantum capillus-veneris* L. Photochem Photobiol 35:533-536

Kami C, Lorrain S, Hornitschek P, Fankhauser C (2010) Light-regulated plant growth and development. In: Marja CPT (ed) Current Topics in Developmental Biology, vol. 91. Academic Press, Oxford, UK, pp 29-66

Kaul KN, Mitra GC, Tripathi BK (1962) Responses of *Marchantia* in aseptic culture to well-known auxins and antiauxins. Ann Bot 26:447-466

Komatsu A, Terai M, Ishizaki K, Suetsugu N, Tsuboi H, Nishihama R, Yamato KT, Wada M, Kohchi T (2014) Phototropin encoded by a single-copy gene mediates chloroplast photorelocation movements in the liverwort *Marchantia polymorpha*. Plant Physiol 166:411-427

Kreh W (1909) Über die Regeneration der Lebermoose. Halle, Druck von E. Karras

Kubota A, Ishizaki K, Hosaka M, Kohchi T (2013) Efficient *Agrobacterium*-mediated transformation of the liverwort *Marchantia polymorpha* using regenerating thalli. Biosci Biotechnol Biochem 77:167-172

Kubota A, Kita S, Ishizaki K, Nishihama R, Yamato KT, Kohchi T (2014) Co-option of a photoperiodic growth-phase transition system during land plant evolution. Nat Commun 5:3668

Liu H, Liu B, Zhao C, Pepper M, Lin C (2011) The action mechanisms of plant cryptochromes. Trends Plant Sci 16:684-691

López-Juez E, Dillon E, Magyar Z, Khan S, Hazeldine S, de Jager SM, Murray JA, Beemster GT, Bögre L, Shanahan H (2008) Distinct light-initiated gene expression and cell cycle programs in the shoot apex and cotyledons of *Arabidopsis*. Plant Cell 20:947-968

Mittmann F, Brücker G, Zeidler M, Repp A, Abts T, Hartmann E, Hughes J (2004) Targeted knockout in *Physcomitrella* reveals direct actions of phytochrome in the cytoplasm. Proc Natl Acad Sci USA 101:13939-13944

Nakazato T, Kadota A, Wada M (1999) Photoinduction of spore germination in *Marchantia polymorpha* L. is mediated by photosynthesis. Plant Cell Physiol 40:1014-1020

Necker NJd (1774) Physiologia Museorum. Manhemii, Schwan, C.F.

Neff MM, Fankhauser C, Chory J (2000) Light: an indicator of time and place. Genes Dev 14:257-271

Nishihama R, Kohchi T (2013) Evolutionary insights into photoregulation of the cell cycle in the green lineage. Curr Opin Plant Biol 16:630-637

Okada S, Fujisawa M, Sone T, Nakayama S, Nishiyama R, Takenaka M, Yamaoka S, Sakaida M, Kono K, Takahama M, Yamato KT, Fukuzawa H, Brennicke A, Ohyama K (2000) Construction of male and female PAC genomic libraries suitable for identification of Y-chromosome-specific clones from the liverwort, *Marchantia polymorpha*. Plant J 24:421-428

Prigge MJ, Bezanilla M (2010) Evolutionary crossroads in developmental biology: *Physcomitrella patens*. Development 137:3535-3543

Rickett HW (1920) Regeneration in Sphaerocarpos Donnellii. Bull Torrey Bot Club 47:347-357

Riou-Khamlichi C, Menges M, Healy JMS, Murray JAH (2000) Sugar control of the plant cell cycle: differential regulation of *Arabidopsis* D-type cyclin gene expression. Mol Cell Biol 20:4513-4521

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9:671-675

Schostakowitsch W (1894) Ueber die Reproductions- und Regenerationserscheinungen bei den Lebermoosen. Flora 79:350-384

Soni R, Carmichael JP, Shah ZH, Murray JA (1995) A family of cyclin D homologs from plants differentially controlled by growth regulators and containing the conserved retinoblastoma protein interaction motif. Plant Cell 7:85-103

Su YS, Lagarias JC (2007) Light-independent phytochrome signaling mediated by dominant GAF domain tyrosine mutants of Arabidopsis phytochromes in transgenic plants. Plant Cell 19:2124-2139

Sugano SS, Shirakawa M, Takagi J, Matsuda Y, Shimada T, Hara-Nishimura I, Kohchi T (2014) CRISPR/Cas9-mediated targeted mutagenesis in the liverwort *Marchantia polymorpha* L. Plant Cell Physiol 55:475-481

Sugimoto K, Jiao Y, Meyerowitz EM (2010) *Arabidopsis* regeneration from multiple tissues occurs via a root development pathway. Dev Cell 18:463-471

Valanne N (1966) The germination phases of moss spores and their control by light. Ann Bot Fenn 3:1

Vöchting H (1885) Über die regeneration der Marchantieen. Jahrbücher für wissenschaftliche Botanik 16:367-414

Wada M (1985) Photoresponses in cell cycle regulation. Proc R Soc Edinb Biol 86:231-235

Wada M, Hayami J, Kadota A (1984) Returning dark-induced cell cycle to the beginning of G1 phase by red light irradiation in fern *Adiantum* protonemata. Plant Cell Physiol 25:1053-1058

Wada M, Kadota A (1989) Photomorphogenesis in lower green plants. Annu Rev Plant Physiol Plant Mol Biol 40:169-191

Xiong Y, McCormack M, Li L, Hall Q, Xiang C, Sheen J (2013) Glucose-TOR signalling reprograms the transcriptome and activates meristems. Nature 496:181-186

Figure legends

Fig. 1 Time-lapse observation of regeneration from thallus explant of *M. polymorpha*. A basal fragment obtained from a 14-day-old thallus of Tak-1 was placed on solid medium containing 1 % sucrose and incubated under continuous white light. Photographs were taken with 1-h intervals, and only the selected time points are shown. *Bar* 2 mm. The complete series of images can be seen in Supplemental Movie 1.

Fig. 2 SEM observation of the initial stages in regeneration. Basal fragments obtained from 10-day-old thalli of Tak-1 were placed on solid media containing 1 % sucrose and incubated under continuous white light. SEM images were taken immediately (**a**), 24 h (**b**), 36 h (**c**), 48 h (**d**, **e**), 72 h (**f**), and 96 h (**g**–**i**) after excision. **a**–**g** ventral-side views; **h**, **i** dorsal-side views; **d** midrib region; **e** thallus blade region. Magnified images for the boxed area in **h** is shown in **i**. Apical side of each fragment is shown upside. *Arrowheads* shallow grooves likely showing recent cell division marks, *arrows* slime papillae, *ellipses* air pores, *mr* midrib region, *pr* pegged rhizoid, *sr* smooth rhizoid, *tb* thallus blade region. *Bars* 100 μm (**a**–**c**, **f**–**i**), 50 μm (**d**, **e**).

Fig. 3 Flow cytometric analysis of DNA contents of *M. polymorpha* thallus. Propidium iodide-stained nuclei from a rosette leaf of Arabidopsis and basal explants of *M. polymorpha* thalli were analyzed by flow cytometry. Histograms of fluorescence intensity are shown.

Fig. 4 Timing of S-phase entry during regeneration under various light conditions. Basal explants of Tak-1 thalli were incubated on solid media without (**a**–**d**) or with (**e**–**h**) 1 % sucrose under continuous white light (cW), in the dark (Dark), under intermittent irradiation of R light (R cycle), or under intermittent irradiation of R and FR light (R/FR cycle). See Supplemental Fig. S4 for the details of the light conditions used. At 0 h, 12 h, 24 h, and 36 h after excision, explants were incubated with 10 μM EdU for 4 h in liquid media of the same kinds under the same light conditions as before. EdU-incorporated DNAs were visualized (pseudo-colored in red) as described in Materials and methods. All DNAs were stained with DAPI (pseudo-colored in blue). Ventral areas around the

midrib, as indicated by the abundance of rhizoids (*r*) and their bases or initials (*rb*), both of which were non-specifically stained with DAPI, are shown with the apical side up. *Bars* 100 μm.

Fig. 5 Effects of sugar and light conditions on regeneration. Ten-day-old thalli of wild type (**a**, **b**) and $_{pro}EF{:}MpPHY^{Y241H}$ transgenic plants (**c**, **d**; two independent lines, #2 and #8) were cut to obtain basal explants. The explants were incubated for 14 days on solid media without (**a**, **c**) or with (**b**, **d**) 1 % sucrose under cW, Dark, R cycle, or R/FR cycle. Apical side of each explant is shown upside. For some cases, obliquely angled images (*Ob*) are displayed to show the upward elongation of the regenerants. *Bars* 2 mm.

Fig. 6 Cell morphology in mature regenerants. Regenerants grown for 14 days after excision in the absence of sucrose under the R cycle (**a**–**c**) and the R/FR cycle (**d**–**f**). **a**, **d** Light microscopic images. **b**, **c**, **e**, **f** SEM images. **c**, **f** Montages of two images. Boxed regions in **a** and **d** are shown in **b** and **e**, and those in **b** and **e** are shown in **c** and **f**, respectively. *Ob* obliquely angled images, *r* rhizoids, *sc* scales. *Bars* 1 mm (**a**, **d**); 500 μm (**b**, **e**); 100 μm (**c**, **f**). **g**–**i** Quantification of cell surface area (**g**), cell length (**h**), and aspect ratio (**i**) shown by box-and-whisker plots. The cells present in the boxed regions in **c** and **f** were used for quantification. *Middle bars* median values, *boxes* interquartile (first to third quartiles) ranges, *top and bottom bars* 1.5× the interquartile ranges, *circles* outliers. *P* values were determined by Student's t-test. *n.s.* not significant. n = 50 (**g**, **i**), n = 181 (**h**, R cycle), n = 106 (**h**, R/FR cycle).

Fig. 7 Effects of R and FR light on cell division and elongation in sporelings. **a** Morphology of sporelings grown in the presence of 0.01 % glucose under the R or R/FR cycle for 10 d. *Bar* 100 μm. **b**–**d** Number of cells (**b**) and branches (**c**) that were contained in each sporeling, and length of cells (**d**) shown by violin plots. The width reflects data density. *White dot* median values, *thick black lines* interquartile ranges, *thin black lines* 1.5× the interquartile ranges. n = 65 (**b**, R cycle), n = 48 (**b**, R/FR cycle), n = 224 (**d**, R cycle), n = 217 (**d**, R/FR cycle).

Fig. 8 Schematic model for photoregulation of regeneration in *M. polymorpha*. The horizontal flow connected by broken-line arrows represents processes that could take place during regeneration. Excision would cause at least two events, sending wounding signals and removal of the apex. While contribution by the former is not clear (*question mark*), the latter, probably through a relief from inhibition by the apex, is critical (see text). These events may trigger changes in the differentiation status of ventral epidermal cells to sporelings or spores. Red light promotes cell cycle re-entry of these cells via production of sugar by photosynthesis and activation of the phytochrome (Pr-to-Pfr conversion), the latter of which also regulates cell shape by modulating the balance between directional and non-directional growth.

Supplemental Fig. S1 Apical-basal polarity in regeneration. **a** Schematic illustration of excision. A thallus of *M. polymorpha* (*thin green line*) was excised with a scalpel along the red broken lines. *Thick green line* midrib. **b**, **c** Micrographs of explants. Both apical and basal explants (**b**) were incubated for 5 days on sugar-free medium (**c**). *Arrows* regenerants. *Bars* 2 mm.

Supplemental Fig. S2 Schematic illustrations of excision. Pink-shaded fragments were used for the experiments shown in Figs. 2, S3, S5 (**a**) and Figs. 4–6, S7 (**b**). The excision pattern in **a** was used for SEM analyses to make sure that only one midrib is included in an explant.

Supplemental Fig. S3 SEM observation of the ventral side of a thallus and explant. **a**, **b** Ventral view of a 12-day-old thallus. Magnified view of the boxed region in **a** is shown in **b**. The dotted line marks a typical excision position. *pr* pegged rhizoid, *sc* scale, *sr* smooth rhizoid. **c** Ventral view of an explant 72 h after excision. *Bars* 500 μm (**a**, **c**), 100 μm (**b**).

Supplemental Fig. S4 Light irradiation patterns for R and R/FR cycle conditions used in this study.

Supplemental Fig. S5 SEM observation of initial stages in regeneration under various light conditions. Explants were grown for 96 h in the dark (**a**, **d**) or under R (**b**, **e**) or R/FR cycle (**c**, **f**) in the

absence (**a**–**c**) or presence (**d**–**f**) of 1 % sucrose. Ventral-side views are presented. Apical side of each explant is shown upside. *Bars* 100 μm.

Supplemental Fig. S6 An amino-acid sequence alignment of GAF domains. The alignment of GAF domains in PHY from *M. polymorpha* and PHYB from Arabidopsis was constructed using the MUSCLE program [Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792-1797] implemented in the Geneious software (version 6.1.8; Biomatters; http://www.geneious.com/) with default parameters. The conserved tyrosine residue that was substituted to histidine is marked by triangle. *Black shade* identical residues.

Supplemental Fig. S7 Comparison of morphology of regenerants grown under R or R/FR cycle. Explants of 10-day-old thalli were incubated on sugar-free media under R (**a**, **b**) or R/FR cycle (**c**, **d**) for 2 weeks. **a**, **c** Dorsal, side, and ventral views of regenerants. **b**, **d** Transverse sections of agar-embedded regenerants dissected approximately along the broken lines in **a** and **c**. *Red arrow* air chamber. *Bars* 200 μm (**a**, **c**), 100 μm (**b**, **d**).

Supplemental Movie 1 Complete series of time-lapse observation of regeneration for 5 days from a thallus explant of *M. polymorpha*. A basal fragment obtained from a 14-day-old thallus of Tak-1 was placed on solid medium containing 1 % sucrose and incubated under continuous white light. Photographs were taken with 1-h intervals.

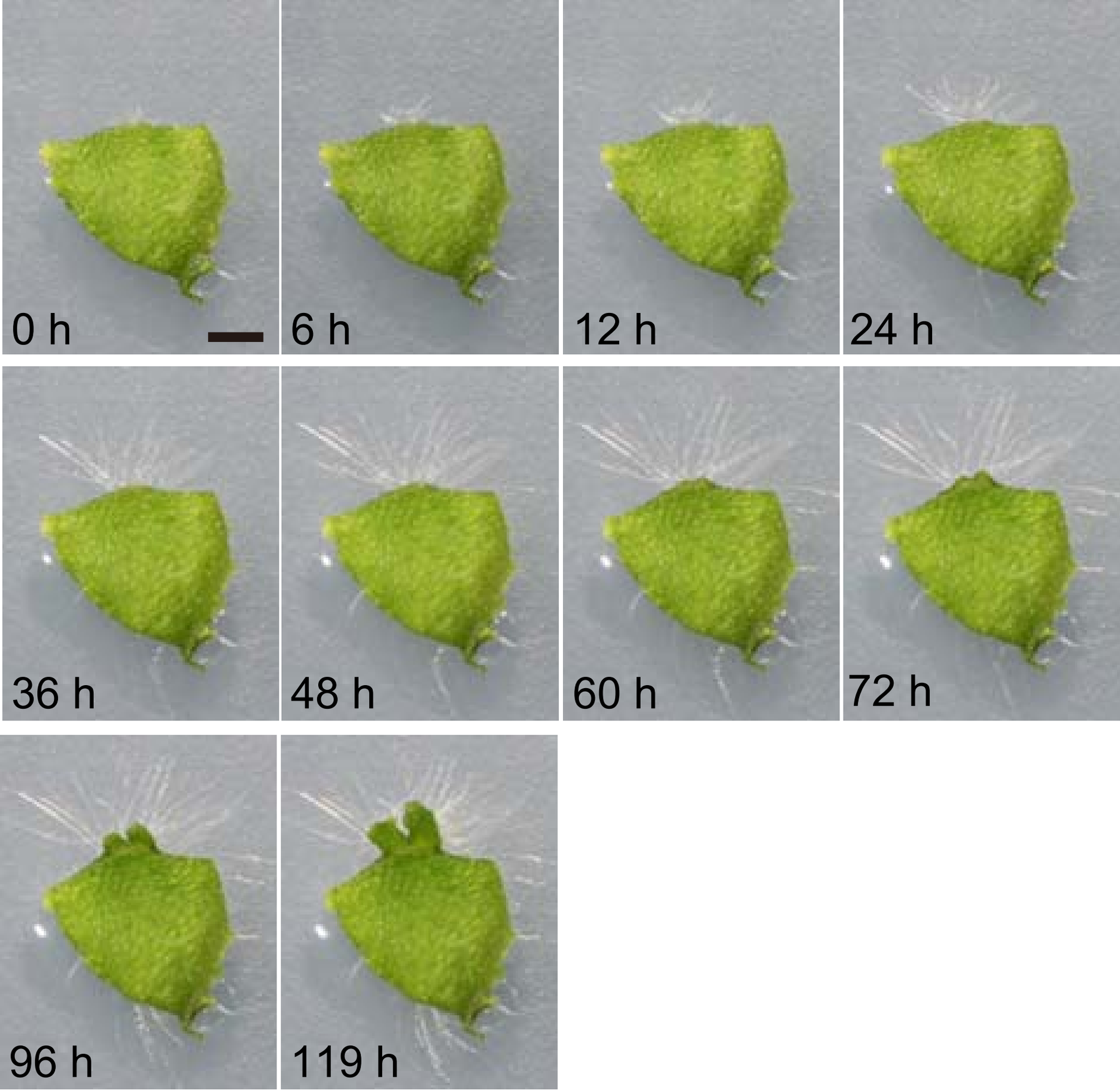
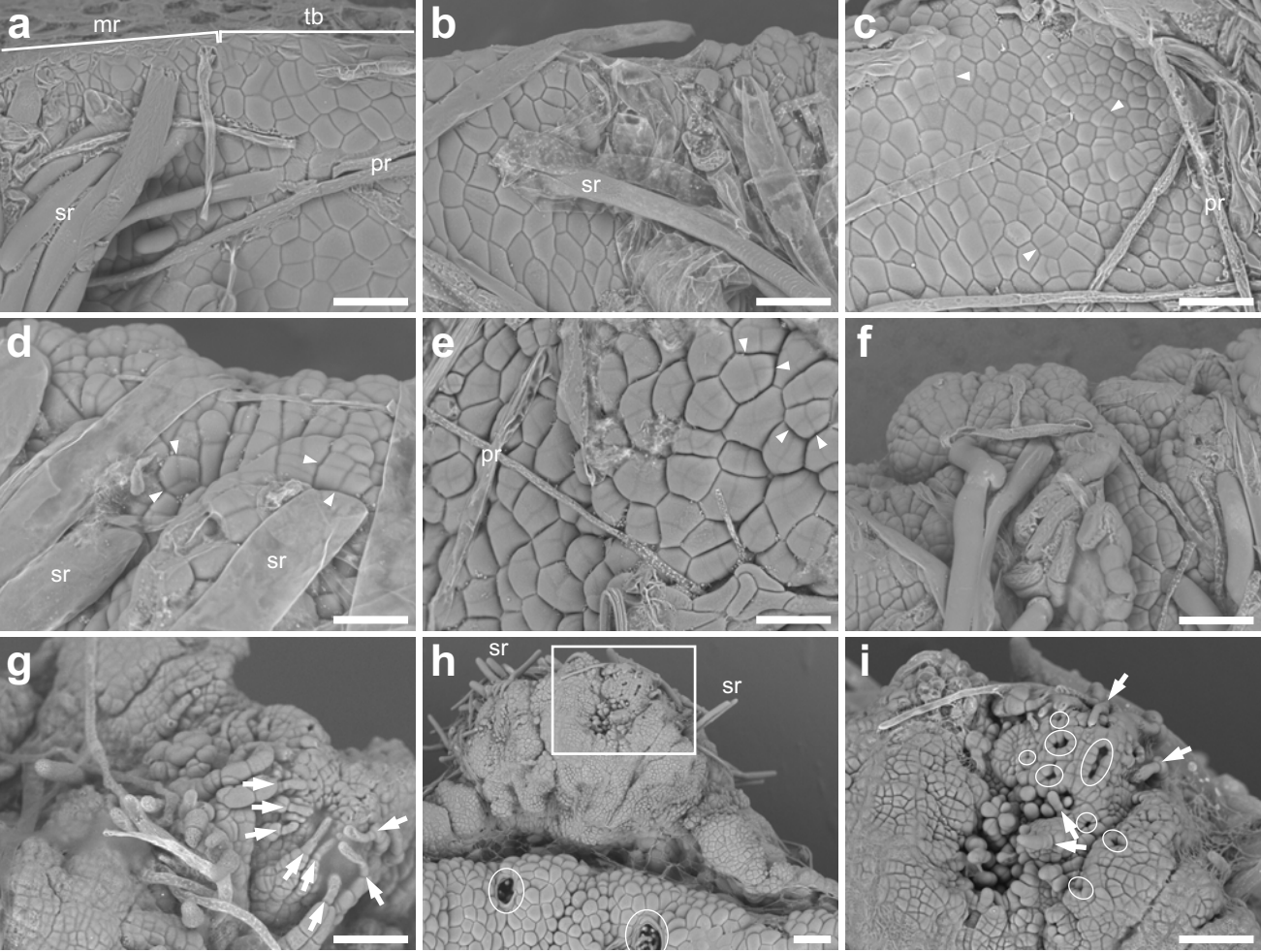


Fig. 1 Nishihama et al.



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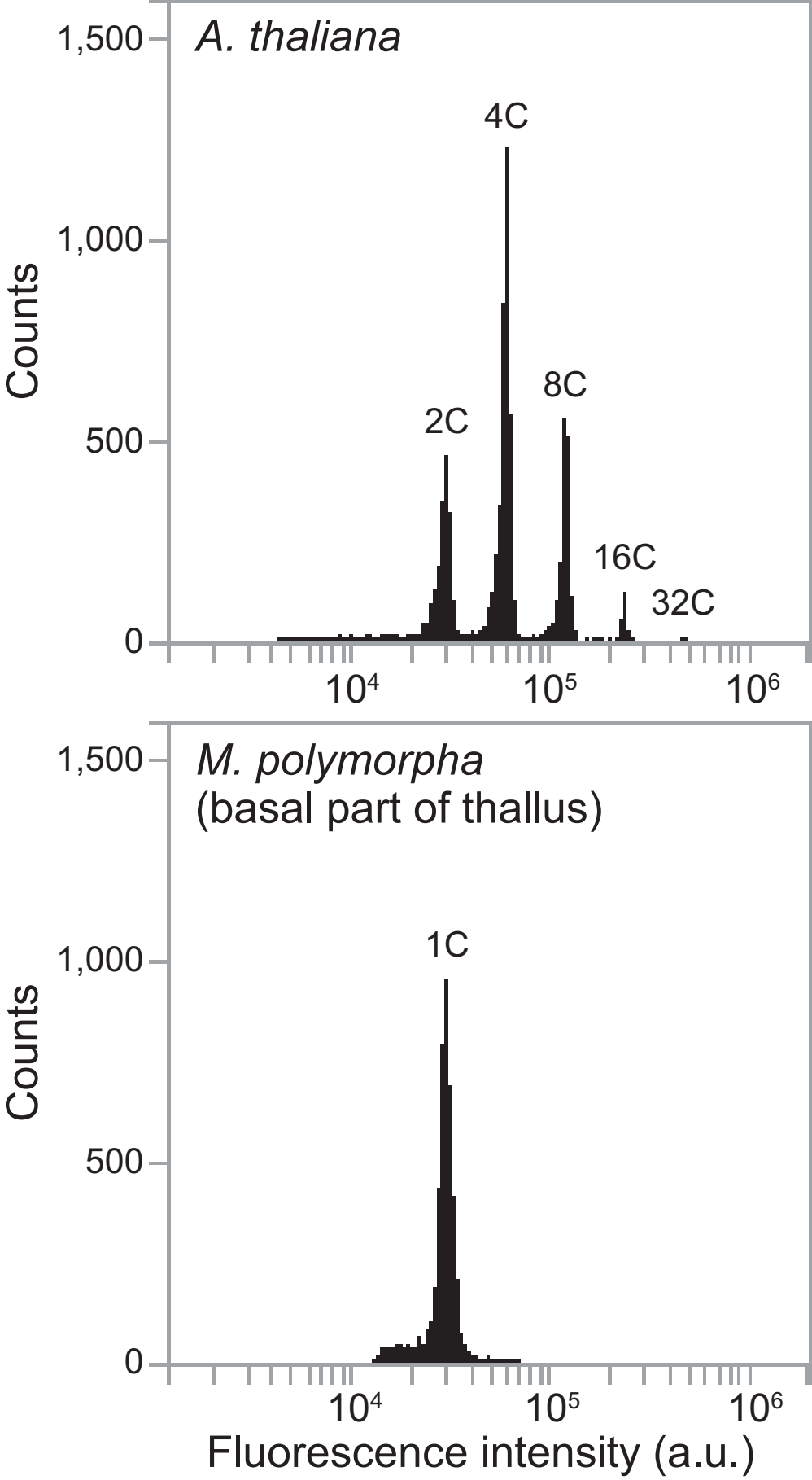

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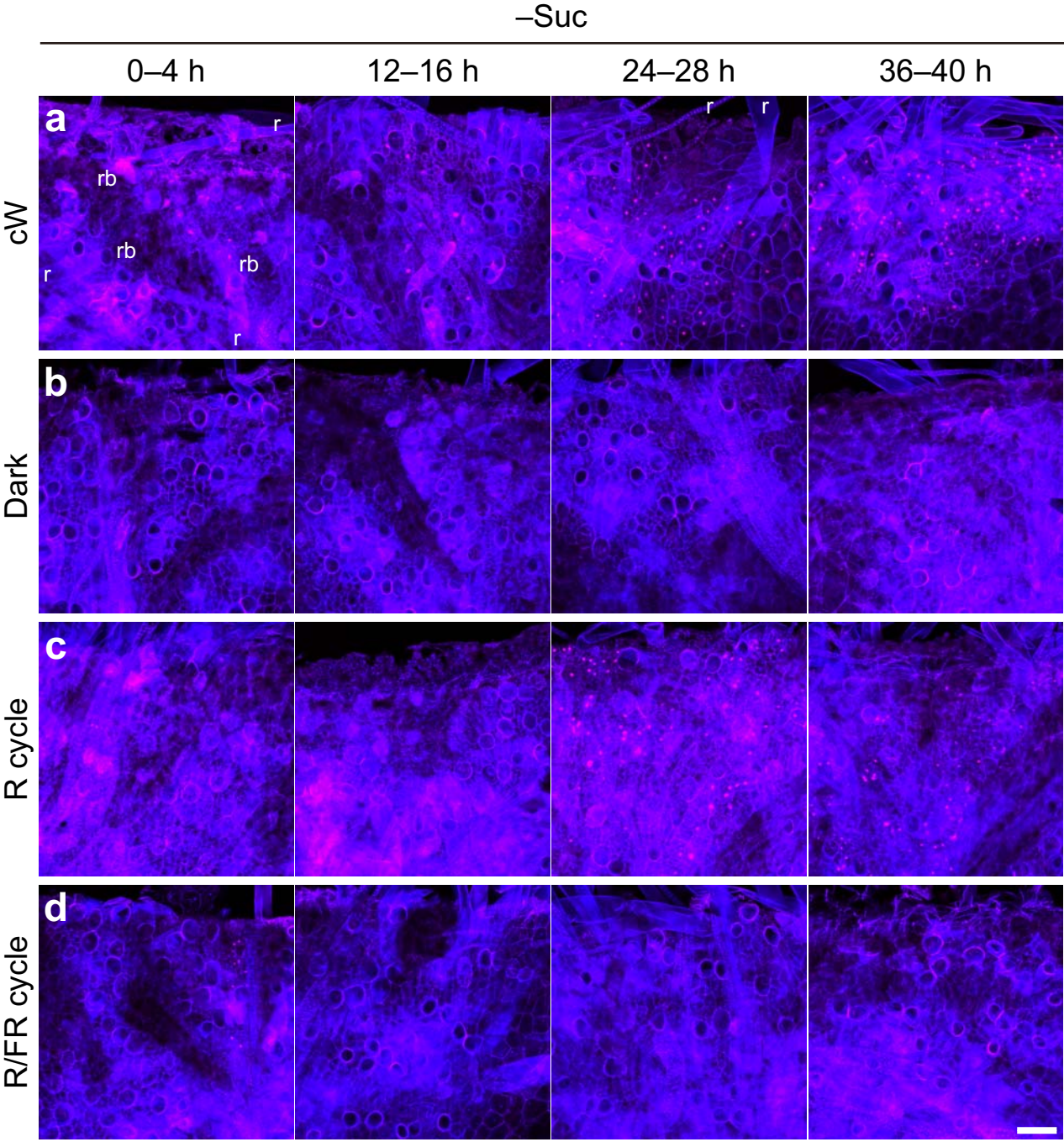

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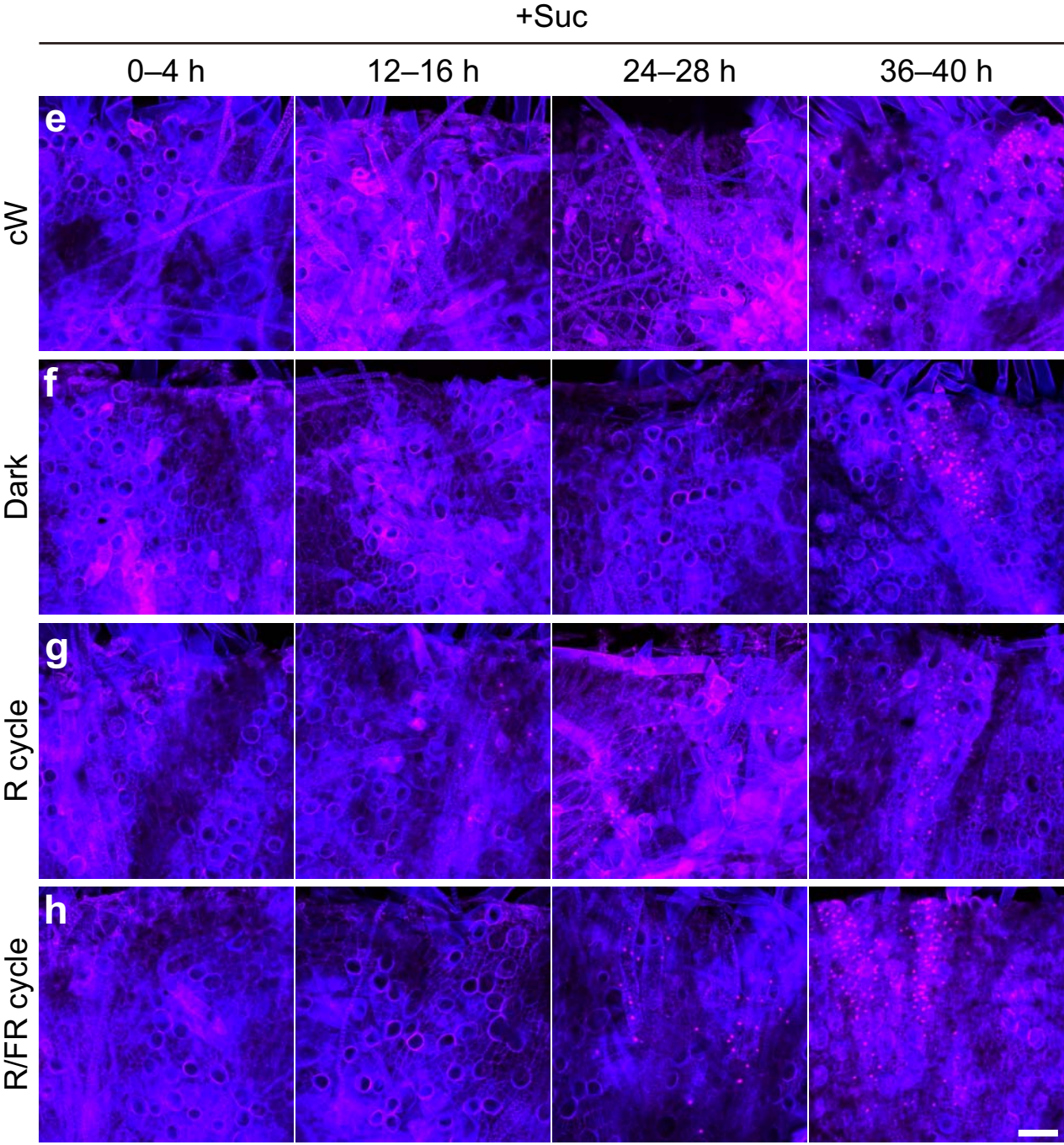

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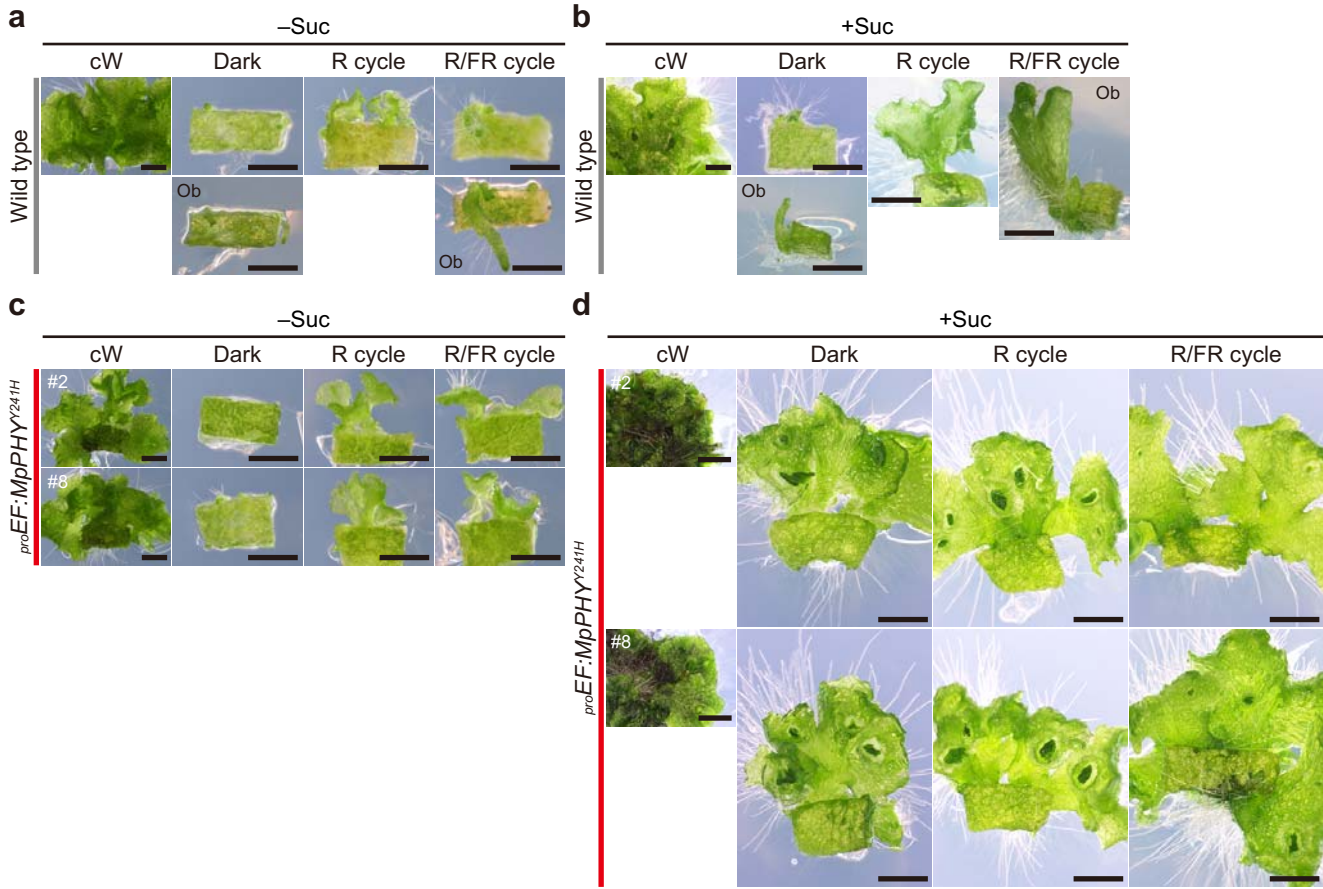

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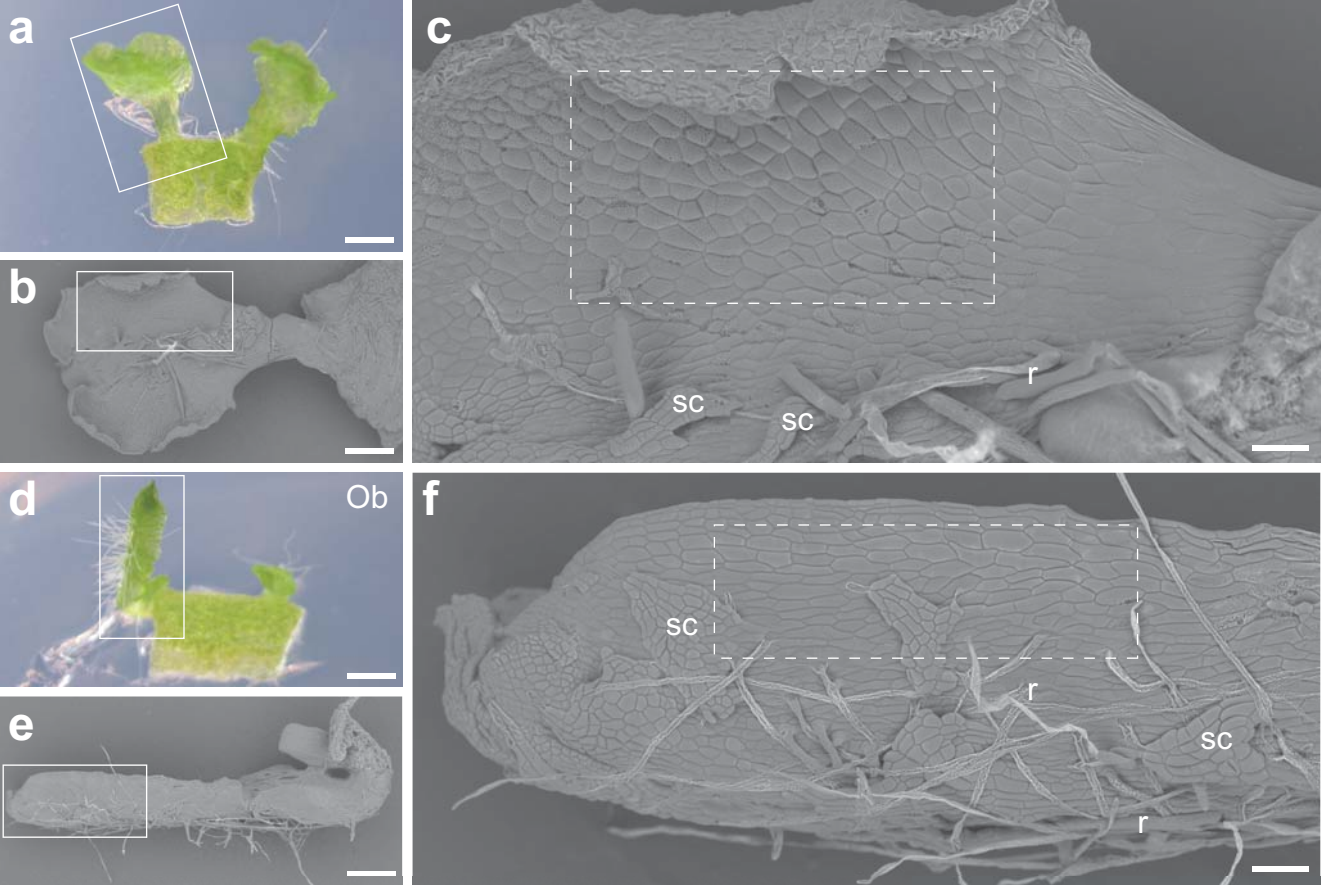

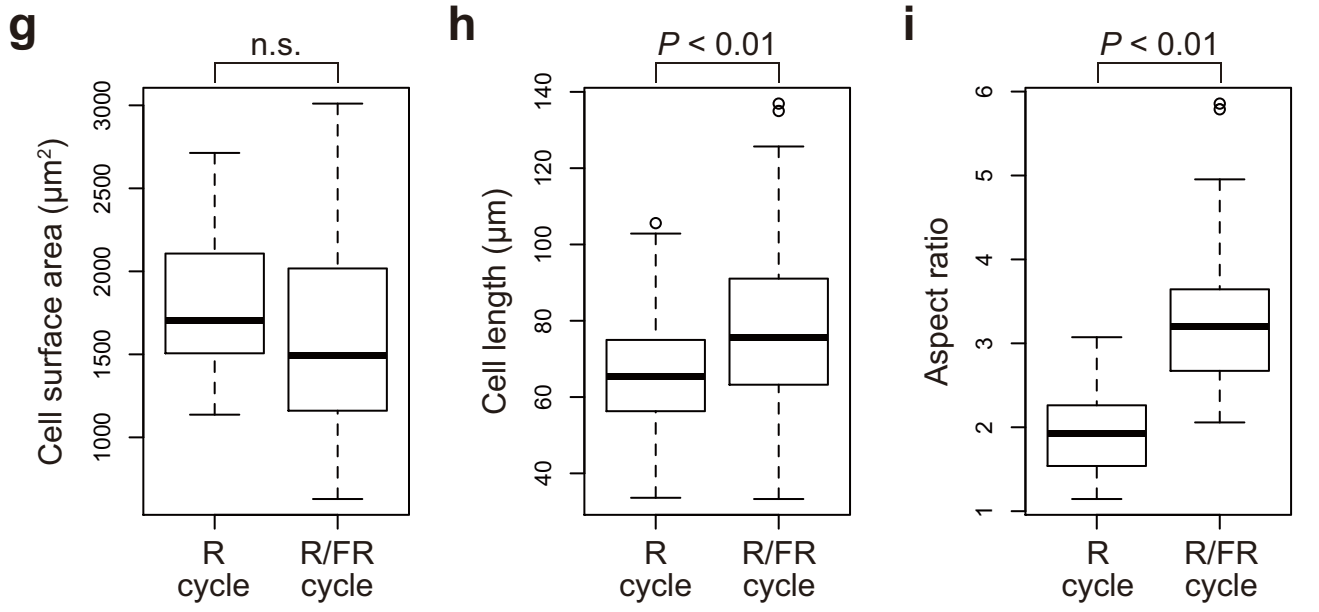

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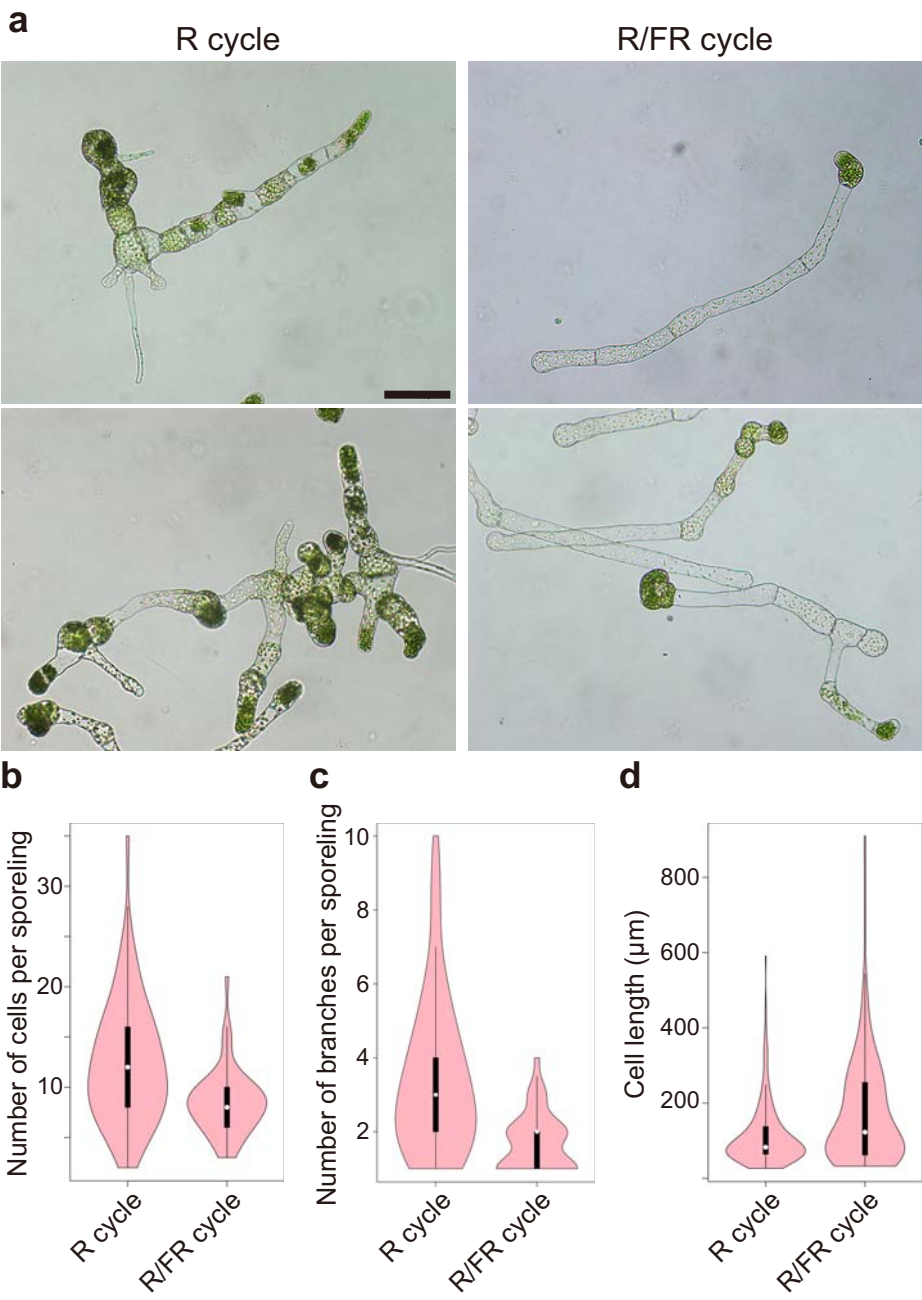
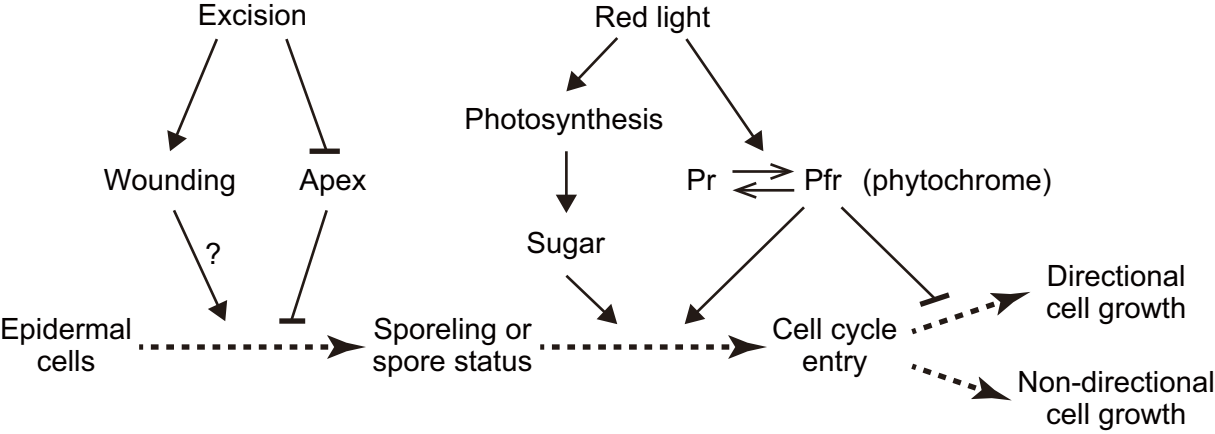


Fig. 7 Nishihama et al.


Excision
Red light
Photosynthesis
Wounding
Apex
Pr
Pfr
(phytochrome)
?
Sugar
Directional
cell growth
Epidermal
cells
Sporeling or
spore status
Cell cycle
entry
Non-directional
cell growth

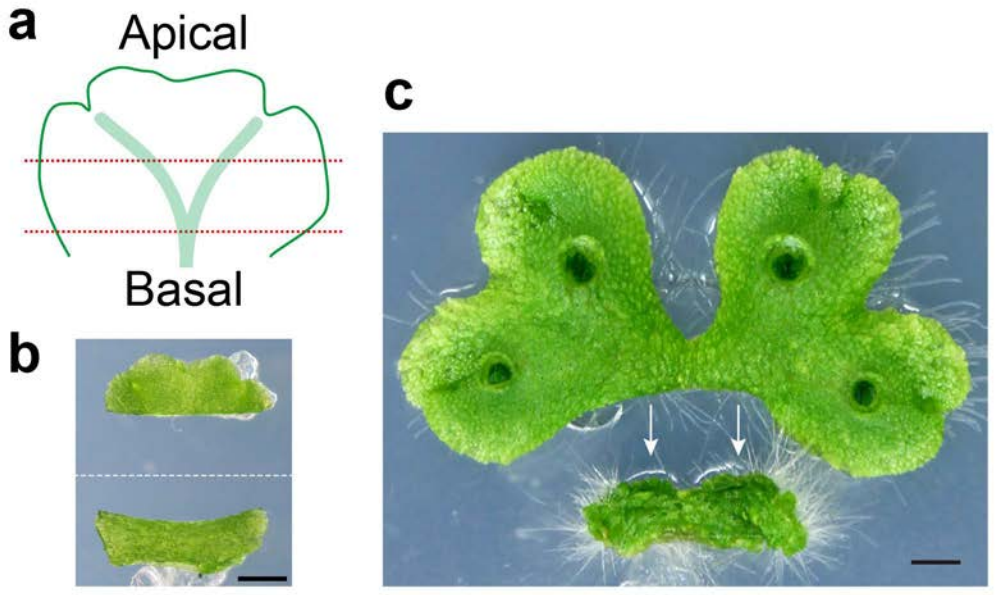


Fig. S1 Apical-basal polarity in regeneration. **a** Schematic illustration of excision. A thallus of *M. polymorpha* (*thin green line*) was excised with a scalpel along the red broken lines. *Thick green line* midrib. **b**, **c** Micrographs of explants. Both apical and basal explants (**b**) were incubated for 5 days on sugar-free medium (**c**). *Arrows* regenerants. *Bars* 2 mm.

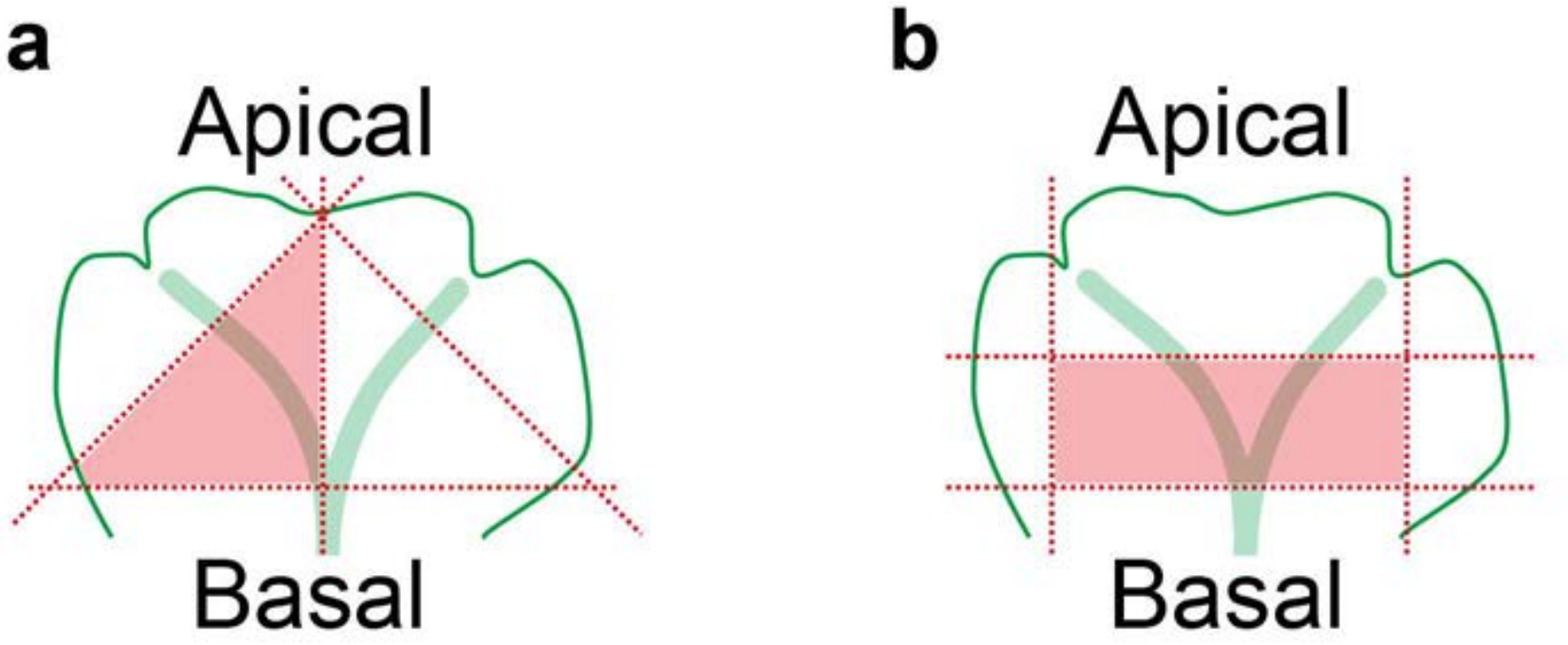


Fig. S2 Schematic illustrations of excision. Pink-shaded fragments were used for the experiments shown in Figs. 2, S3, S5 (**a**) and Figs. 4–6, S7 (**b**). The excision pattern in **a** was used for SEM analyses to make sure that only one midrib is included in an explant.

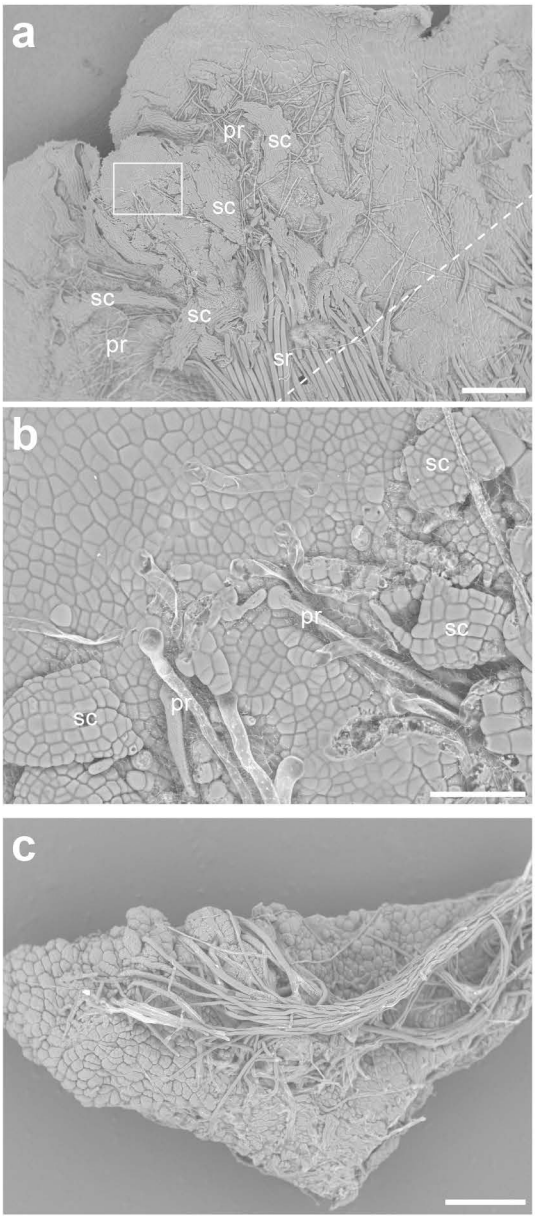


Fig. S3 SEM observation of the ventral side of a thallus and explant. **a**, **b** Ventral view of a 12-day-old thallus. Magnified view of the boxed region in a is shown in **b**. The dotted line marks a typical excision position. *pr* pegged rhizoid, *sc* scale, *sr* smooth rhizoid. **c** Ventral view of an explant 72 h after excision. *Bars* 500 μm (**a**, **c**), 100 μm (**b**).

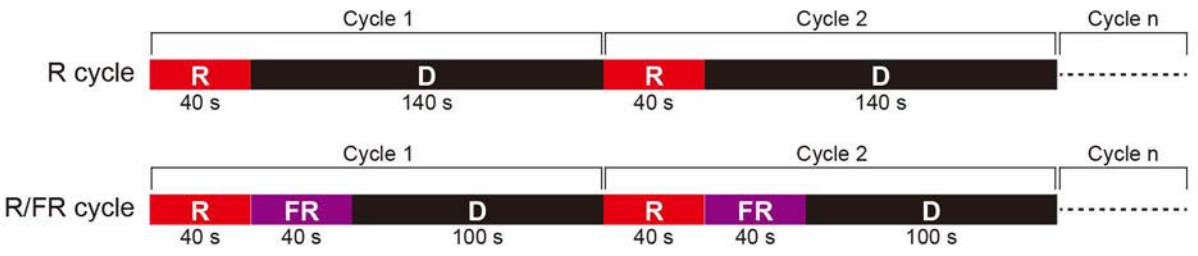


Fig. S4 Light irradiation patterns for R and R/FR cycle conditions used in this study.

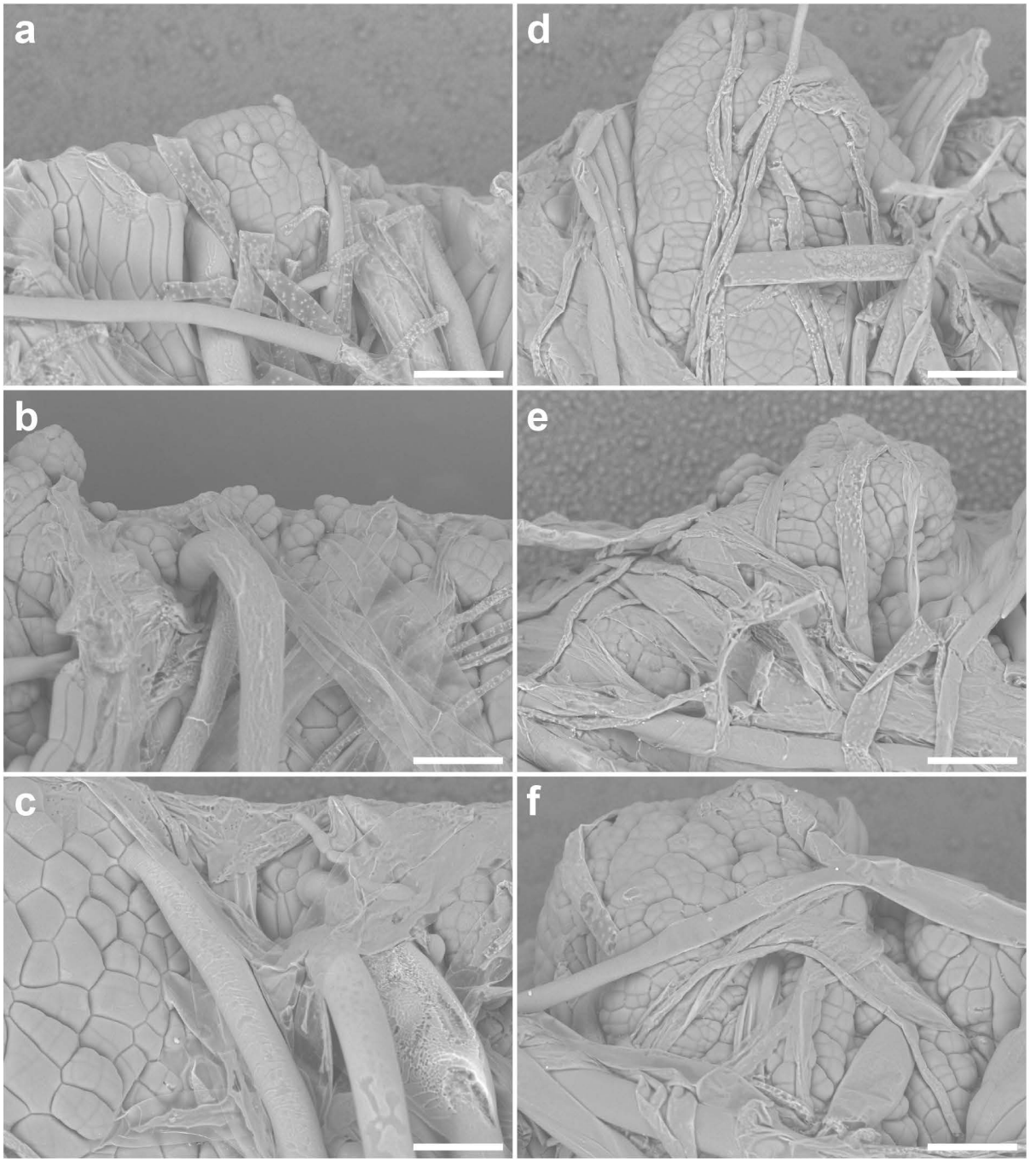


Fig. S5 SEM observation of initial stages in regeneration under various light conditions. Explants were grown for 96 h in the dark (**a**, **d**) or under R (**b**, **e**) or R/FR cycle (**c**, **f**) in the absence (**a**–**c**) or presence (**d**–**f**) of 1 % sucrose. Ventral-side views are presented. Apical side of each explant is shown upside. *Bars* 100 μm.

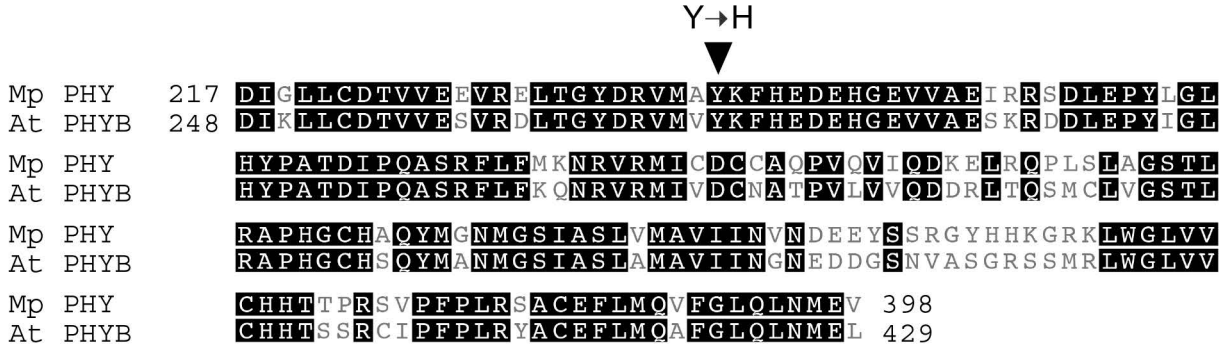


Fig. S6 An amino-acid sequence alignment of GAF domains. The alignment of GAF domains in PHY from *M. polymorpha* and PHYB from Arabidopsis was constructed using the MUSCLE program [Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792-1797] implemented in the Geneious software (version 6.1.8; Biomatters; http://www.geneious.com/) with default parameters. The conserved tyrosine residue that was substituted to histidine is marked by triangle. *Black shade* identical residues.

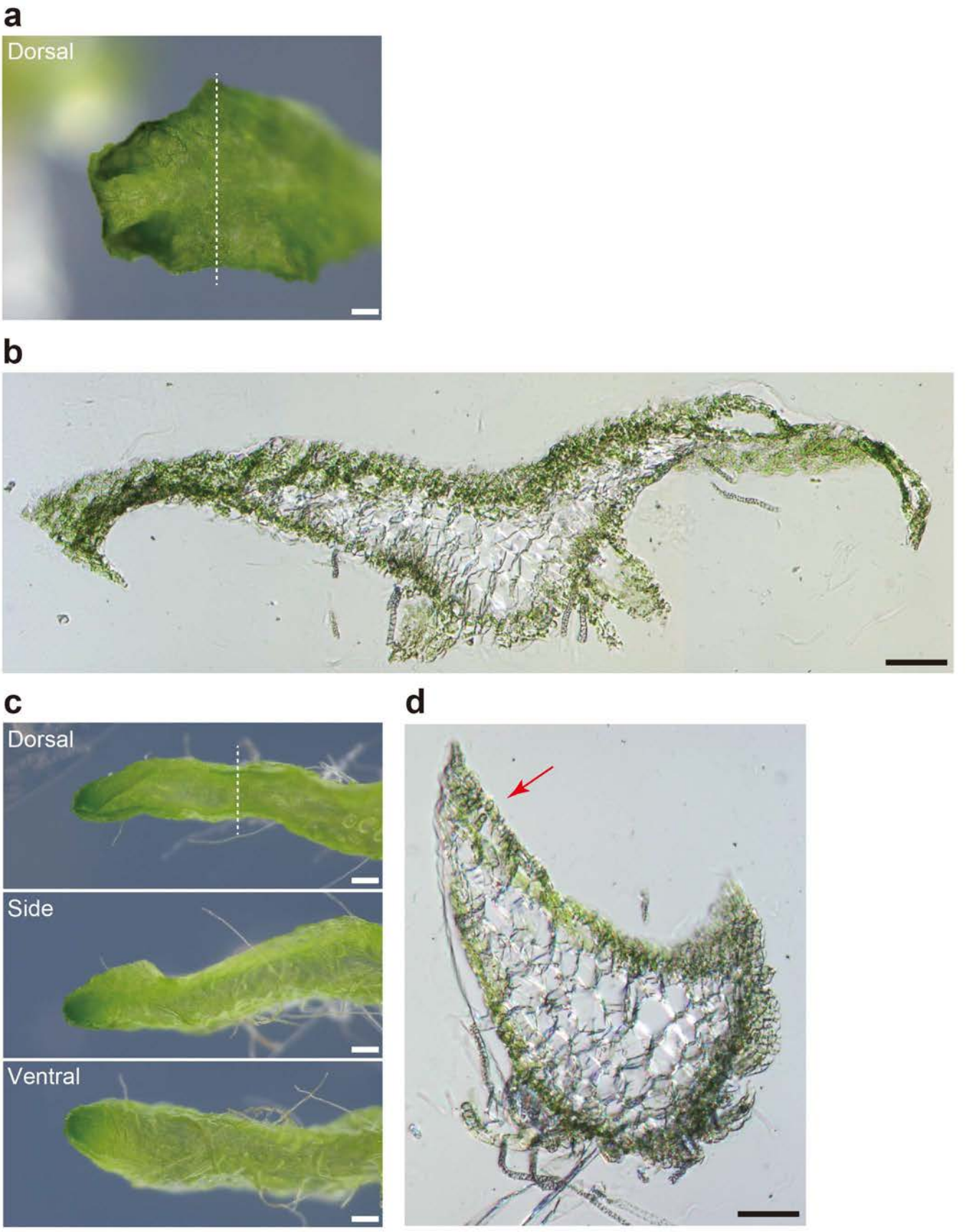


Fig. S7 Comparison of morphology of regenerants grown under R or R/FR cycle. Explants of 10-day-old thalli were incubated on sugar-free media under R (**a**, **b**) or R/FR cycle (**c**, **d**) for 2 weeks. **a**, **c** Dorsal, side, and ventral views of regenerants. **b**, **d** Transverse sections of agar-embedded regenerants dissected approximately along the broken lines in **a** and **c**. *Red arrow* air chamber. *Bars* 200 μm (**a**, **c**), 100 μm (**b**, **d**).