Original Paper

A glycogen synthase kinase 3-like kinase MpGSK regulates cell differentiation in *Marchantia polymorpha*

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Abstract Plants precisely coordinate the balance between cell proliferation and differentiation to ensure the continuous development. In *Arabidopsis thaliana*, members of glycogen synthase kinase 3 (GSK3) family, which are highly conserved serine/threonine protein kinases among eukaryotes, play important roles in regulating cell proliferation and differentiation during various developmental processes. However, functional roles of GSK3s in the plant lineages except angiosperms remain to be elucidated. Here, we utilized a model liverwort, *Marchantia polymorpha*, for studies of GSK3, because it has a single GSK3-like kinase, MpGSK. When *M. polymorpha* was treated with a chemical compound, bikinin, which is known as a specific inhibitor for GSK3-like kinases, growth and morphologies were altered with an expansion of the meristematic region. Similarly, *Mpgsk* loss-of-function mutants accumulated undifferentiated cell mass with no differentiated tissues. By contrast, overexpression of MpGSK reduced the size of the meristem region. These results suggest that MpGSK plays important roles as a regulator for the balance between cell differentiation and proliferation in *M. polymorpha*.

Key words: bryophyte, cell differentiation, cell proliferation, glycogen synthase kinase 3, inhibitor.

Introduction

GSK3s are highly conserved serine/threonine protein kinases in eukaryotes (Li et al. 2021; Qi et al. 2013; Yoo et al. 2006). In *Arabidopsis thaliana*, 10 GSK3-like kinases, which are named *A. thaliana* GSK3/SHAGGY-like kinases (AtSKs), play key roles in various biological processes such as developmental regulation and stress responses (Li et al. 2021; Mao et al. 2021). Of those, BRASSINOSTEROID INSENSITIVE 2 (BIN2)/AtSK21 and its two closest homologs BIN2-LIKE1 (BIL1)/AtSK23 and BIL2/AtSK22 are implicated in signaling pathway of a plant hormone brassinosteroid (Li and Nam 2002; Nolan et al. 2020; Yan et al. 2009). In brassinosteroid signaling, BIN2 negatively regulates key transcription factors BRI1-EMS-SUPPRESSOR 1 (BES1) and BRASSINAZOLERESISTANT 1 (BZR1) via

direct phosphorylation (He et al. 2002; Yin et al. 2002). GSK3s also function in the regulation of xylem cell differentiation from cambial cells (Kondo et al. 2014). In an ectopic vascular cell transdifferentiation system Vascular cell Induction culture System Using Arabidopsis Leaves (VISUAL), inhibition of the kinase activities of GSK3s by a specific inhibitor bikinin is critical to facilitate the differentiation of both xylem and phloem cells (Kondo et al. 2015, 2016). In addition, GSK3s are known to regulate the proper differentiation of guard cells and pavement cells in leaves (Gudesblat et al. 2012; Kim et al. 2012).

The roles of GSK3s in developmental regulation and stress responses have been reported in various angiosperms such as rice *Oryza sativa*, cotton *Gossypium hirsutum*, soybean *Glycine max*, barley *Hordeum vulgare* (Li et al. 2021; Mao et al. 2021). Moreover, in moss

Abbreviations: ARFs, AUXIN RESPONSE FACTORS; AtSKs, *A. thaliana* GSK3/SHAGGY-like kinases; BES1, BRI1-EMS-SUPPRESSOR 1; BIL1, BIN2-LIKE1; BIL2, BIN2-LIKE2; BIN2, BRASSINOSTEROID INSENSITIVE 2; BZR1, BRASSINAZOLERESISTANT 1; CYCD, D-type cyclins; DDCZ, dividing and differentiating cell zone; GSK3, glycogen synthase kinase 3; NLS, nuclear localization signal; SCZ, stem cell zone; Tak-1, Takaragaki-1; VISUAL, vascular cell induction culture system using Arabidopsis leaves.

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Physcomitrium (previously called *Physcomitrella*) *patens*, GSK3-like kinase genes, *PpSKs*, are transcriptionally activated in response to osmotic stress (Richard et al. 2005). However, in the plant lineages except angiosperms, the function of GSK3s especially in the cell differentiation processes are poorly understood. Here, we utilized a model liverwort *Marchantia polymorpha* because of their advantages in molecular biological experiments (Bowman et al. 2017; Ishizaki et al. 2016; Kohchi et al. 2021; Shimamura 2016). Treatment of bikinin severely affected the growth and morphogenesis with the expansion of the meristematic region in *M. polymorpha*. In addition, our reverse genetic approaches toward a single *M. polymorpha* GSK3-like kinase, MpGSK, revealed the importance of MpGSK in balancing cell proliferation and differentiation for the well-coordinated thallus morphogenesis. Thus, our findings provide novel information about the function of GSK3s in the land plant lineage.

Materials and methods

Plant materials and culture conditions

The *M. polymorpha* male accession Takaragaike-1 (Tak-1) was used as the wild-type line (Ishizaki et al. 2008; Okada et al. 2000). BC4 spores were obtained by four-time backcrosses of a female Tak-2 line to Tak-1 (Bowman et al. 2017). Wild-type and transgenic plants were cultured on half-strength Gamborg's B5 medium (Gamborg et al. 1968) containing 1% (w/v) agar with 1% (w/v) sucrose under $50\text{--}60\mu\text{mol m}^{-2}\text{s}^{-1}$ continuous white light at 22°C. For β -estradiol-inducible system, gemmae were sown on plates containing $10\mu\text{M}$ β -estradiol.

Construction of transgenic plants

For generation of *proMpCYCD;1:MpCYCD;1^{Nter}-tdTomato-NLS* lines, a genomic region covering from ~5-kb upstream to 141-bp downstream of the initiation codon, which includes a putative PEST motif-coding sequence (aa 1-42), was amplified by PCR using a primer set, MpCYCD_pro1F 5'-caccATGGAA TCGATCAAACGTTG-3' and MpCYCD_PEST_R 5'-GCT ATGCAGAGCCGGATGTAG-3', and cloned in pENTR/D-TOPO. The resulting plasmid was used for LR recombination with pMpGWB316 using LR Clonase II (ThermoFisher) to create pMpGWB316-*proMpCYCD;1:MpCYCD;1* (PEST), which was used for *Agrobacterium*-mediated transformation of BC4 spores (Ishizaki et al. 2008). Transformants were selected on half-strength B5 agar medium containing 1% (w/v) agar, $0.5\mu\text{M}$ chlorsulfuron, and 100mg l^{-1} cefotaxime (Claforan; Sanofi-Aventis). The MpGSK targeting vectors used for genome editing with the CRISPR/Cas9 system were constructed according to Sugano et al. (2018). MpGSK-gRNA-F1 5'-gcaccagcctctcgATATGGCCTCGGCAAGCG gtttagagctagaaA-3' and MpGSK3-gRNA-R1 5'-ttctagctctaa aacCGCTTGCCGAGGCCATATcgagagctgggtgc-3' for guide RNA were incubated together at 95°C for 5 min to enable

annealing. The resultant annealed oligonucleotides containing the target sequence were cloned into pMpGE_En01 digested with *SacI* and *PstI* to construct the entry vector using the In-Fusion HD cloning kit (Clontech or TaKaRa Bio). The sequence between attL1 and attL2 within the entry vector was inserted into the binary vector pMpGE011 by the LR reaction using LR Clonase II. The thallus transformation with the CRISPR/Cas9 system were described previously (Kubota et al. 2013). *Agrobacterium* containing the pMpGE011-MpGSK-gRNA vector was transformed into thalli from Tak-1. Transformants were selected on half-strength B5 agar medium containing 1% (w/v) agar, $0.5\mu\text{M}$ chlorsulfuron, and 100mg l^{-1} cefotaxime. To construct overexpression lines of MpGSK-L kinase negative form (KN) fused Citrine (*proMpEF1 α :MpGSK-LKN-Citrine*), the coding sequence of MpGSK-L was amplified from Tak-1 genomic DNA by PCR using KOD FX Neo (Toyobo) and then cloned into pENTR/D-TOPO (Life Technologies). The entry vector for the MpGSK-LKN (K171R), was constructed by mutagenesis on pENTR/D-TOPO-MpGSK-L using the following primer pair: 5'-GAAACGGTGGCAATCCGGAAG GTGTTGCAG-3' and 5'-CTGCAACACCTTCCGGATTGC CACCGTTTC-3'. The region between attL1 and attL2 within the entry vectors were inserted into the destination vector pMpGWB108 (Ishizaki et al. 2015) by LR reaction using LR clonase II (Life Technologies). *Agrobacterium* containing the pMpGWB108-MpGSK-LKN vector was transformed into thalli from *proMpCYCD;1:MpCYCD;1^{Nter}-tdTomato-NLS* transgenic line and selected on half-strength B5 agar medium containing 1% (w/v) agar, 10mg l^{-1} hygromycin, and 100mg l^{-1} cefotaxime. To construct β -estradiol-inducible MpGSK-L overexpressing lines, the coding sequence of MpGSK-L was amplified from Tak-1 genomic DNA by PCR using KOD FX Neo (Toyobo). The region between attL1 and attL2 within the entry vectors was inserted into the destination vector pMpGWB168 by LR reaction using LR Clonase II. This destination vector contains the β -estradiol-responsible chimeric XVE transcription factor gene (Zuo et al. 2000) that is driven by a promoter region of *M. polymorpha* *E2F* (Mp1g02890) and the *lexA* operator sequences (Zuo et al. 2000) that are located in the upstream of a transgene inserted by LR reaction. The transformation toward gemmalings from Tak-1 were carried out using improved G-AgarTrap method described previously (Tsuboyama et al. 2018). Transformants were selected on half-strength B5 agar medium containing 1% (w/v) agar, $10\mu\text{g ml}^{-1}$ hygromycin B, and 100mg l^{-1} cefotaxime.

Scanning electron microscopy

Sample preparation and observation were performed as described previously with a slight modification (Furuya et al. 2018). The samples were fixed in FAA [50% (v/v) ethyl alcohol, 2.5% (w/v) glacial acetic acid, and 2.5% (w/v) formalin] and dehydrated using a graded ethanol series and isoamyl acetate, and dried using a JCPD-5 critical point dryer (JEOL, Tokyo, Japan). Dried samples were coated with platinum using a JEC-3000FC sputter-coater (JEOL). Images were captured using a

JSM-6510LV scanning electron microscope (JEOL, Tokyo, Japan).

5-ethynyl-2'-deoxyuridine (EdU) and cell wall staining

To assess the cell proliferation activity, the Click-iT EdU Imaging Kit (Life Technologies) was used as described previously with slight modifications (Furuya et al. 2021; Naramoto et al. 2019; Nishihama et al. 2015). Three- or five-day-old plants after gemma germination were soaked in half-strength Gamborg's B5 medium with 10 μ M EdU at 22°C for 1 h under continuous white light. Thalli were fixed in FAA and were degassed by vacuum infiltration. EdU in the fixed samples were detected with Alexa Fluor 555-azide following the manufacturer's protocol. For clearing and cell wall staining, samples were treated with the ClearSee solution [10% (w/v) xylitol, 15% (w/v) sodium deoxycholate, and 25% (w/v) urea] with 0.02% (v/v) SCRI Renaissance 2200 (Renaissance Chemicals, Selby, UK) (Kurihara et al. 2015). After replacing with the ClearSee solution, samples were kept for two additional days or more. For microscopic observations, samples

were mounted on glass slides with the ClearSee solution; Alexa Fluor 555 and SCRI Renaissance 2200 fluorescence images were obtained using the confocal laser scanning microscope (Olympus FLUOVIEW FV1000, Tokyo, Japan) at excitation and detection wavelengths of 559 and 570–670 nm for Alexa Fluor 555 fluorescence, and at 405 and 425–460 nm for SCRI Renaissance 2200 fluorescence, respectively.

Results

Bikinin treatment expands the meristematic region in *M. polymorpha*

A chemical compound bikinin is known to induce the hypocotyl elongation in *A. thaliana* via the inhibition of GSK3s (de Rybel et al. 2009). To investigate the effect of bikinin on *M. polymorpha*, we cultured the wild-type line (Tak-1) on the medium containing various concentrations of bikinin for 10 days after gemma germination. Thalli treated with 25 μ M and 50 μ M bikinin exhibited significantly small and abnormal morphologies compared with that of control condition

Figure 1. Morphology of the bikinin-treated *M. polymorpha*. (A) Morphology of thalli grown from gemmae of the wild-type line (Tak-1) on bikinin containing media for 10 days. (B) The area of thalli grown on the bikinin containing media for 10 days. Data represent mean $\pm$ standard deviation (SD). Gray dots indicate values of each thallus ($n=4-7$). Different lowercase letters indicate significant differences ($p<0.05$; Tukey–Kramer test). (C–F) Morphology of plants grown from gemmae of *proMpCYCD;1:MpCYCD;1^{Nter}-tdTomato-NLS* on the bikinin containing media for 10 days. Top views of thalli are shown as bright-field images (C, D). tdTomato signals and autofluorescence of each plant (C, D) are shown as merged images (E, F). Red and white indicate tdTomato signals and autofluorescence, respectively. (G, H) EdU-containing nuclei in the apical region cultured with (H) or without (G) 50 μ M bikinin for 5 days were visualized by EdU staining. Plants were treated with EdU-containing liquid medium for 1 h. Z-series fluorescence images were obtained using the confocal laser scanning microscope. Maximum projection images of are shown. EdU-containing nuclei are shown as red foci. White dotted lines indicate the edge of thalli. (K) The numbers of EdU-containing foci were measured based on the fluorescence microscope images and are shown in box-and-whisker plots. The upper and lower limits indicate the first and third interquartile ranges, respectively, and the middle bar indicates the median. Whiskers indicate 1.5 $\times$ the interquartile ranges. Gray dots indicate values of each notch ($n=8$). Significant differences were examined by Student's *t*-test. (I, J) S-phase nuclei and cell morphologies in the apical region cultured with (J) or without (I) 50 μ M bikinin for 5 days were visualized by EdU staining and SCRI Renaissance 2200 staining. EdU-containing nuclei are shown as red foci. Cell walls stained by SCRI Renaissance 2200 are shown in white. A single slice of fluorescence image was obtained using the confocal laser scanning microscope. Scale bars: 2 mm (A), 1 mm (C–F), 100 μ m (G–J).

(Figure 1A, B; Supplementary Figure S1). In general, the activity and location of cell proliferation is important to ensure proper tissue morphogenesis. During the thallus growth, cell proliferation was mainly active in the meristematic region, apical notch (Kohchi et al 2021; Shimamura 2016). To visualize the cell proliferation activity, we monitored the expression level of D-type cyclins (CYCD), which are responsible for triggering the G1/S transition (Meijer and Murray 2000; Nieuwland et al. 2009). In a reporter line harboring an *M. polymorpha* CYCD (MpCYCD;1), in which an N-terminal stretch of MpCYCD;1 fused to nuclear localization signal (NLS)-tagged fluorescent protein tdTomato was expressed under the control of MpCYCD;1 promoter (*proMpCYCD;1:MpCYCD;1^{Nter}-tdTomato-NLS*), tdTomato signals were preferentially detected around the apical notches under mock condition (Figure 1C, E). On the other hand, bikinin treatment expanded the domain of strong tdTomato signals to band-shaped areas (Figure 1D, F). In addition, we also assumed the cell proliferation activity using EdU staining, in which a thymidine analog EdU is incorporated into newly synthesized DNA during DNA replication. To focus on the effect of bikinin treatment in the early developmental stage, we used thalli cultured with or without 50 μ M bikinin for 5 days after gemma germination (Figure 1G–K). Although the number of EdU-containing nuclei in the apical regions under bikinin condition were identical to that under mock condition, the area of EdU signals under bikinin condition was expanded more broadly than those under mock condition (Figure 1G–J). Moreover, bikinin-treated thalli showed more opened apical notches compared with mock-treated plants (Figure 1I, J). And apical- and/or subapical-like cells in the apical notch of the bikinin-

treated plants seem to be increased than that of mock-treated plants (Figure 1I, J). These results suggest that bikinin treatment induces expansion of the meristematic region.

M. polymorpha has a single GSK3-like kinase ortholog

Bikinin inhibits the kinase activities of GSK3-like kinases in *A. thaliana* (de Rybel et al. 2009). A single GSK3-like kinase, MpGSK (Mp7g04170; Supplementary Figure S2A), was identified in the genome of *M. polymorpha* (MarpolBase genome database [v5.1: <http://marchantia.info>]; Mecchia et al. 2021; Montgomery et al. 2020). MpGSK has a highly conserved serine/threonine protein kinase domain including the activation loop and the TREE domain for the proteasome-mediated protein degradation (Peng et al. 2008), as other plant GSK3s possess (Supplementary Figure S2B). In the *M. polymorpha* genome database, two alternative transcripts in the MpGSK locus were annotated (Figure 2A). Although both transcripts encode proteins involving the kinase domain, Mp7g04170.2 has an expanded N-terminal region compared with Mp7g04170.1 (Figure 2A). Thus, we named Mp7g04170.1 and Mp7g04170.2 as MpGSK-S and MpGSK-L, respectively (Figure 2A, B).

Mpgsk mutants produced undifferentiated cell mass

To uncover the role of MpGSK in *M. polymorpha*, we generated the *Mpgsk* genome-edited lines using the CRISPR/Cas9 system (Sugano et al. 2018). Target site was designed in the coding region of both alternative transcripts (Figure 2A). We obtained two genome-edited lines, *Mpgsk-1^{se}* and *Mpgsk-2^{se}* (Figure 2C,

Figure 2. Construction and observation of *Mpgsk* genome-edited lines. (A) Estimated two transcriptional variants of MpGSK3, MpGSK-L (Mp7g04170.2) and MpGSK-S (Mp7g04170.1). The coding regions and the untranslated regions are indicated as orange and gray boxes, respectively. Blue arrowhead indicates the location of target sites of gRNAs for the genome editing. (B) Protein structure of MpGSK-L. Green box indicates kinase domain. Blue arrowhead indicates the location of gRNA target sites. (C) A part of genomic sequences of MpGSK in the genome-edited lines. The wild type (Tak-1) sequence is shown together with the PAM sequence (orange) and the target sequence (underline). Red residues indicate the start codon for MpGSK-S. The genome-edited line *Mpgsk-1^{se}* have a 1 bp insertion, while *Mpgsk-2^{se}* has a 2 bp substitution and a 5 bp deletion respectively (blue). (D) Partial amino acid sequences of MpGSK in the WT (Tak-1) and two genome-edited lines. Red indicates the translational start site for MpGSK-S. The genome-edited lines have frame shift mutation (blue). (E–G) Morphology of explants of *Mpgsk-1^{se}* (E) and *Mpgsk-2^{se}* (F and G). (H) Scanning electron microscopy image of *Mpgsk-2^{se}*. Scale bars: 1 mm (E–G), 100 μ m (H).

D), both of which resulted in frameshifts in protein translation of MpGSK (Figure 2D). Hereafter, these mutants were used as loss-of-function mutants of MpGSK for further studies. Both *Mpgsk-1^{se}* and *Mpgsk-2^{se}* formed an undifferentiated cell mass phenotype (Figure 2E–H). Moreover, these mutants exhibited no typical differentiated dorsal or ventral structures such as air pore, gemma cup, and ventral scale (Figure 2E–H). These morphological phenotypes indicate that MpGSK has important roles for cell differentiation in the thallus development. To investigate whether the kinase activity of MpGSK is critical for cell differentiation, we created two overexpression lines of MpGSK-L mutated at Lys171 in the ATP-binding site to Arg for converting to the kinase negative form (KN), *proMpEF1a:MpGSK-LKN-Citrine-1* and *proMpEF1a:MpGSK-LKN-Citrine-2*, to expect the dominant-negative effects (Supplementary Figure S3). Both lines exhibited thin layers of tissues lacking typical differentiated structures such as air chambers, air pores, and gemma cups (Supplementary Figure S3). Moreover, the overexpression lines generated behind the *proMpCYCD;1:MpCYCD;1^{Nter}-tdTomato-NLS*

background, showed expanded tdTomato signals in the apical region without typical notch shape similar to the phenotypes of the bikinin-treated plants (Supplementary Figure S3D). These suggest that the kinase activity of MpGSK is important for the regulation of the cell differentiation.

MpGSK overexpression repressed the cell proliferative activity

To investigate the function of MpGSK in more detail, we generated two stable transgenic lines harboring β -estradiol-inducible MpGSK-L overexpressing lines, *proMpE2F:XVE>>MpGSK-L-1* and *proMpE2F:XVE>>MpGSK-L-2* (Figure 3A–D). When these inducible MpGSK-overexpressing lines were cultured with β -estradiol for 6 days, both lines showed reduced growth and morphological defects and exhibited opened apical notches compared with mock treatment condition (Figure 3A–D). Then to assume the cell proliferation activity, we measured the number of EdU-containing nuclei in the apical notches. The number of EdU signals in the apical notches of

Figure 3. Morphology and visualization of S-phase nuclei in the β -estradiol-inducible MpGSK overexpression lines. (A–D) Morphology of thalli grown from gemmae of the β -estradiol-inducible MpGSK overexpression lines, *proMpE2F:XVE>>MpGSK-L-1* (A and B), and *-2* (C and D). Transgenic plants were treated with (B and D) or without (A and C) 10 μ M β -estradiol for 6 days. (E–F) EdU-containing nuclei in *proMpE2F:XVE>>MpGSK-L-1* cultured with (F) or without (E) 10 μ M β -estradiol for 3 days were visualized by EdU staining. Plants were treated with EdU-containing liquid medium for 1 h. Z-series fluorescence images were obtained using the confocal laser scanning microscope. Maximum projection images of are shown. EdU-containing nuclei are shown as red foci. White dotted lines indicate the edge of thalli. (G) The numbers of EdU-containing foci were measured based on the fluorescence microscope images and are shown in box-and-whisker plots. The upper and lower limits indicate the first and third interquartile ranges, respectively, and the middle bar indicates the median. Whiskers indicate $1.5\times$ the interquartile ranges. The black dot indicates an outlier. Gray dots indicate values of each notch ($n=12$). Significant differences were examined by Student's *t*-test (** $p<0.01$). (H–K) S-phase nuclei and cell morphologies in *proMpE2F:XVE>>MpGSK-L-1* cultured with (J, K) or without (H, I) 10 μ M β -estradiol for 3 days were visualized by EdU staining and SCRI Renaissance 2200 staining. EdU-containing nuclei are shown as red foci. Cell walls stained by SCRI Renaissance 2200 are shown in white. A single slice of fluorescence image was obtained using the confocal laser scanning microscope. White dashed boxes in (H) and (J), which contain the stem cell zone (SCZ) and the dividing and differentiating cell zone (DDCZ), are magnified in (I) and (K), respectively. Yellow dots indicate apical and/or subapical cells. Scale bars: 1 mm (A–D), 100 μ m (E, F, H, J), 50 μ m (I, K).

proMpE2F:XVE>>MpGSK-L-1 cultured with β -estradiol for 3 days was significantly reduced compared with that cultured under mock condition (Figure 3E–G). These results suggest that overexpression of MpGSK decreased the cell proliferation activity in the apical notches. The apical notch consists of the stem cell zone (SCZ) including apical cells, and the dividing and differentiating cell zone (DDCZ) (Kohchi et al. 2021). Next, we observed the cell morphology in the apical notch in the *proMpE2F:XVE>>MpGSK-L-1* (Figure 3H–K). Under mock condition, DDCZ is composed of relatively small cells with high cell proliferation activity (Figure 3H, I), while estradiol treatment decreased the population of small cells in the DDCZ of the *proMpE2F:XVE>>MpGSK-L-1* (Figure 3J, K). These results suggest that overexpression of MpGSK inhibits cell proliferation activity in the DDCZ.

Discussion

Effect of bikinin treatment in *M. polymorpha*

GSK3-like kinases of seed plants are classified into four subgroups (Group I–IV) (Qi et al. 2013). In *A. thaliana*, bikinin treatment induced alteration of seedling morphology such as elongation of hypocotyl via inactivation of all members of group I GSK3s (AtSK11, AtSK12, and AtSK13) and group II GSK3s (BIN2, BIL1, and BIL2), and a part of group III GSK3s (AtSK32) (de Rybel et al. 2009). For the inhibitory mechanism of GSK3 activity by bikinin, several amino acid residues (including Met115 and Tyr117 in BIN2) located in the ATP binding pocket are important (de Rybel et al. 2009). The predicted amino acid sequence (LEY) in this region of MpGSK differs from the sequence (MEY) in *A. thaliana* group II GSK3s including BIN2 (Supplementary Table S1). However, the LEY sequence is found in the amino acid sequences of group I and III, most of which can be the targets of bikinin (Supplementary Table S1; de Rybel et al. 2009). These results suggest the possibility that bikinin is also effective to MpGSK. Indeed, the phenotypes of bikinin-treated plants and *Mpgsk* mutants were similar in terms of enhancing undifferentiated state of cells. Further studies at the molecular level will provide clues to uncover the functional mechanism of bikinin in *M. polymorpha*.

MpGSK regulates the cell proliferation and differentiation for the proper thallus development

Mpgsk genome-edited lines of *M. polymorpha* suppressed thallus development to produce undifferentiated cell mass. Moreover, these mutants lacked differentiated tissues, indicating that MpGSK has an essential role in controlling the cell differentiation (Figure 2). Conversely, overexpression of MpGSK led to the reduction of cell proliferation in the DDCZ (Figure 3). Considering that

Mpgsk mutants inhibit cell differentiation to maintain undifferentiation state, it is possible that reduction of DDCZ by MpGSK overexpression is caused by promotion of cell differentiation. Taken together, it can be concluded that MpGSK has important roles as a regulator for balancing cell differentiation and proliferation. In *A. thaliana*, GSK3-like kinases contribute to the cell differentiation in the various developmental processes, such as the vascular and stomatal development (Gudesblat et al. 2012; Kim et al. 2012; Kondo et al. 2015, 2014, 2016). Thus, in both *A. thaliana* and *M. polymorpha*, GSK3-like kinases have general roles in the regulation of cell differentiation, suggesting that the functions of GSK3-like kinases as regulator for the cell differentiation are likely evolutionarily conserved.

It is known that the plant hormone auxin acts in the differentiation processes in *M. polymorpha* (Eklund et al. 2015; Kato et al. 2015; Kohchi et al. 2021). The loss-of-function mutants of an auxin biosynthesis enzyme MpTAA (Eklund et al. 2015) or overexpressors of a dominant auxin signaling repressor (non-degradable mutant of AUXIN/INDOLE-3-ACETIC ACID; Kato et al. 2015) exhibit undifferentiated cell mass phenotype, similarly to *Mpgsk* mutants. In *A. thaliana*, group II BIN2 phosphorylates several members of AUXIN RESPONSE FACTORS (ARF2, ARF7, and ARF19) and another group II BIL1 phosphorylates ARF5/MP (Cho et al. 2014; Han et al. 2018; Vert et al. 2008). Transcription factors BES1 and its homolog BZR1 are well-known substrates of GSK3s such as BIN2 in *A. thaliana* (He et al. 2002; Kondo et al. 2014; Yin et al. 2002). Recently, it was reported that the ortholog of *A. thaliana* BES1 and BZR1, MpBES1, is involved in the regulation of cell proliferation and cell differentiation in *M. polymorpha* (Mecchia et al. 2021). MpBES1 phosphorylation by MpGSK was much weaker than the phosphorylation of BES1/BZR1 by BIN2 (Mecchia et al. 2021). Moreover, the interaction between MpBES1 and MpGSK was not stronger as that between BES1 and BIN2 or MpGSK in yeast two-hybrid system (Mecchia et al. 2021). However, the phenotypes of both *Mpbes1* and *Mpgsk* show same trend in terms of repression of the cell differentiation, suggesting that MpBES1 is one of possible candidates for substrate of MpGSK in *M. polymorpha*. Further biochemical and genetical analyses will help uncover the molecular mechanism of MpGSK in the regulation of cell differentiation status.

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