A plant-specific DYRK kinase DYRKP coordinates cell morphology in *Marchantia polymorpha*

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Abstract

Dual-specificity tyrosine phosphorylation-regulated kinases (DYRKs) are activated via the auto-phosphorylation of conserved tyrosine residues in their activation loop during protein translation, and they then phosphorylate serine/threonine residues on substrates. The DYRK family is widely conserved in eukaryotes and is composed of six subgroups. In plant lineages, DYRK homologs are classified into four subgroups, DYRK2s, yet another kinase1s, pre-mRNA processing factor 4 kinases, and DYRKPs. Only the DYRKP subgroup is plant-specific and has been identified in a wide array of plant lineages, including land plants and green algae. It has been suggested that in *Arabidopsis thaliana* DYRKPs are involved in the regulation of centripetal nuclear positioning induced by dark light conditions. However, the molecular functions, such as kinase activity and the developmental and physiological roles of DYRKPs are poorly understood. Here, we focused on a sole DYRKP ortholog in the model bryophyte, *Marchantia polymorpha*, MpDYRKP. MpDYRKP has a highly conserved kinase domain located in the C-terminal region and shares common sequence motifs in the N-terminal region with other DYRKP members. To identify the roles of MpDYRKP in *M. polymorpha*, we generated loss-of-function *Mpdyrkp* mutants via genome editing. *Mpdyrkp* mutants exhibited abnormal, shrunken morphologies with less flattening in their vegetative plant bodies, thalli, and male reproductive organs, antheridial receptacles. The surfaces of the thalli in the *Mpdyrkp* mutants appeared uneven and disordered. Moreover, their epidermal cells were drastically altered to a narrower shape when compared to the wild type. These results suggest that MpDYRKP acts as a morphological regulator, which contributes to orderly tissue morphogenesis via the regulation of cell shape.

Keywords Cell shape · Liverwort · Morphogenesis · Protein kinase

Introduction

The dual-specificity tyrosine (Y) phosphorylation-regulated kinase (DYRK) family belongs to the larger Cdk2, MAPK, GSK3, CLK and related kinase family (CMGC), that has been identified in a wide range of animals, plants, fungi, and protists (Aranda et al. 2011; Manning et al. 2002; Varjosalo et al. 2013). Generally, DYRKs are activated via the auto-phosphorylation of a conserved Y residue in the activation loop during protein translation and they then phosphorylate serine/threonine (S/T) residues on substrates. (Lochhead et al. 2005; Soppa and Becker 2015). The DYRK family is composed of six subgroups: DYRK1s, DYRK2s, yet another kinase1s (Yak1s), pre-mRNA processing factor 4 kinases (PRP4Ks), homeodomain-interacting protein kinases (HIPKs), and plant-specific DYRKs (DYRKPs) (Kajikawa et al. 2015). DYRKs in yeast and mammals are well known

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as key regulators of the cell cycle and differentiation (Aranda et al. 2011; Becker 2012; Soppa and Becker 2015). Yak1 in *Saccharomyces cerevisiae*, which is a founding member of the DYRK family, is a negative regulator of proliferation under nutritional stress (Garret and Broach 1989). Mammalian DYRK1A, which is well known as a candidate gene for involvement in Down syndrome, functions in neuronal differentiation (Gwack et al. 2006; Kurabayashi and Sanada 2013). In addition, DYRK2 plays important roles in cell cycle and apoptosis regulation (Nihira and Yoshida 2015; Taira et al. 2007, 2012).

There are only four DYRK subgroups in plant lineages, including land plants and green algae, and these include DYRK2, Yak1, PRP4K, and DYRK. Triacylglycerol accumulation regulator1 (TAR1), a homolog of the Yak subgroup in *Chlamydomonas reinhardtii*, was identified as the gene responsible for the low triacylglycerol (TAG) accumulation mutant that is known to occur in sulfur and nitrogen deficient conditions (Kajikawa et al. 2015). Under nitrogen-deficient conditions, TAR1 also regulates the growth and degradation of chlorophyll and photosynthesis-related proteins. The loss-of-function mutant for *Arabidopsis thaliana* Yak homolog, AtYAK1, showed less sensitivity to the plant hormone abscisic acid (ABA) in the context of seed germination, seedling growth, and stomatal closure, suggesting that AtYAK1 positively regulates the ABA signaling transduction pathway (Kim et al. 2016). In addition, AtYAK1 was also found to interact with LIGHT-REGULATED WD1 (LWD1) and LWD2, which are key regulators of the circadian period (Huang et al. 2017). Indeed, *atyak1* mutants have a longer circadian period length and delayed flowering phenotype than the wild type (Huang et al. 2017). Moreover, AtYAK1 functions downstream of TARGET OF RAPAMYCIN (TOR) kinase, which is involved in the regulation of meristem activity and cell differentiation during root development (Barrada et al. 2019; Forzani et al. 2019; Xiong et al. 2013).

Unlike the other subgroups, the DYRK subgroup is only found in plant genomes (Kajikawa et al. 2015; Schulz-Raffelt et al. 2016). In *A. thaliana*, four DYRKs (DYRK1, DYRK2, DYRK3, and DYRK4) are encoded in the genome (Iwabuchi et al. 2019). These DYRKs were shown to interact with ANGUSTIFOLIA (AN) in the yeast two-hybrid system, and it is suggested that this complex contributes to dark-induced centripetal nuclear positioning via the alignment of actin filaments (Iwabuchi et al. 2019). In addition, DYRK in *C. reinhardtii* negatively regulates the accumulation of starch and oil synthesis in low energy status conditions (Schulz-Raffelt et al. 2016). However, the functions of DYRKs in other contexts, including their morphological, developmental, and evolutionary aspects, requires further elucidation.

Here, we focused on the liverwort *Marchantia polymorpha*, which is part of the bryophyte (Bowman et al. 2017).

M. polymorpha is a well-established model plant with various experimental advantages such as a short life cycle, ease of culturing and crossing, a low level of genetic redundancy, and available genetic information and gene manipulation systems (Ishizaki et al. 2016; Kohchi et al. 2021; Shimamura 2016). Indeed, *M. polymorpha* has a single DYRK ortholog, MpDYRK. We generated genome-edited mutants, *Mpdyrk*, which exhibited defects in their thallus morphology as they showed shrinking and less flattening. Moreover, epidermal cells in *Mpdyrk* mutants were drastically altered to narrower shapes compared to the wild type. Our findings provide novel knowledge regarding the role of DYRKs in terms of morphology.

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). Wild-type and transgenic plants were cultured on half-strength Gamborg's B5 medium (Gamborg et al. 1968) containing 1 % (w/v) agar with or without 1 % (w/v) sucrose or soil under 50–60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ continuous white light at 22 °C. To induce the reproductive phase, gemmalings were cultured on soil under continuous 50–60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ white light supplemented with 20–40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ far-red light (730 nm) irradiation (VBL-T600-1, Valore).

Construction of transgenic plants

The MpDYRK targeting vectors used for genome editing with the CRISPR/Cas9 system were constructed according to Sugano et al. (2018). MpDYRK-gRNA-F1 5'-CTC GGT CCT ATT TTC AGG GGC A-3' and MpDYRK-gRNA-R1 5'-AAA CTG CCC CTG AAA ATA GGA C-3' for guide RNA-1, and MpDYRK-gRNA-F2 5'-CTC GAT CGT ACA CAG GAA AAA C-3' and MpDYRK-gRNA-R2 5'-AAA CGT TTT TCC TGT GTA CGA T-3' for guide RNA-2 were incubated together at 95 °C for 5 min, then cooled to 85 °C at a rate of $-2\text{ }^{\circ}\text{C s}^{-1}$, held at 85 °C for 1 min, then further cooled to 25 °C at $-0.2\text{ }^{\circ}\text{C s}^{-1}$ to enable annealing. The resultant annealed oligonucleotides containing the target sequence were inserted into the *BsaI* site of pMpGE_En03 to construct the entry vector. The sequence between attL1 and attL2 within the entry vector was inserted into the binary vector pMpGE010 by the LR reaction using LR clonase II (Life Technologies). The vector used for the complementation analysis was constructed based on the method of Ishizaki et al. (2015). The genomic sequences of the promoter region (5-kb upstream of the start codon) and

terminator region (1-kb downstream of the stop codon) of MpDYRK_P were amplified from the Tak-1 genomic DNA using the following primers: MpDYRK_Ppro-F 5'-TGG ATC CGG TAC CGA ATT CGG ACT TGG AAC ATC TAG C-3' and MpDYRK_Ppro-R, 5'-CGA GTG CGG CCG CGA ATT GGC TGG ATA TGA TTG AGA TCA C-3' for the promoter, and MpDYRK_Pterm-NotI-F 5'-TAT CTC CGC GCG GCC GTG AGA GTT ATC TTT CAA TC-3' and MpDYRK_Pterm-NotI-R1 5'-ATC TCG AGT GCG GCC TGT GGT TTA CCA TAT AAT TC-3' for the terminator. The stop codon in the MpDYRK_Pterm-NotI-F primer is underlined in the above sequence. The longest MpDYRK_P coding sequence corresponding to the splicing variant Mp3g19940.1, was amplified from Tak-1 thalli cDNA using the following primers: MpDYRK_PPORF-NotI-F 5'-ATC CAG CCA ATT CGC GGC CAT GGC GGA TTC GGT CAA TGC C-3' and MpDYRK_PPORF-NotI-R 5'-AGA TAT CTC GAG TGC GGC GCG GAG ATA GGT TCA TAT GG-3'. The MpDYRK_P promoter region (hereafter *pro*MpDYRK_P) was cloned into pENTR1A digested with *Eco*RI using the SLICE method (Motohashi 2015). Then, the MpDYRK_P ORF was inserted into the *Not*I site of pENTR1A-*pro*MpDYRK_P. During this cloning, the *Not*I site on the 3' side of the ORF was preserved, while the *Not*I site on the 5' side of the ORF was not. Finally, the MpDYRK_P terminator region (hereafter MpDYRK_Pterm) was inserted into the *Not*I site of the pENTR1A-*pro*MpDYRK_P:MpDYRK_P ORF plasmid. The *pro*MpDYRK_P:MpDYRK_P ORF:MpDYRK_Pterm region between attL1 and attL2 within the resultant entry plasmid was inserted into the destination vector pMpGWB301 (Ishizaki et al. 2015) by LR reaction using LR clonase II to generate the *pro*MpDYRK_P:MpDYRK_P construct. To confirm the introduction of the *pro*MpDYRK_P:MpDYRK_P construct in the transformants, the primers, MpDYRK_PPORFE2-F1 5'-GCA AGG AGG CTT AGT GGA GGT A-3' and MpDYRK_PPORFE2-R1 5'-TCT CCA CTT CCT CGT CAT CAT C-3' were used for PCR screening.

A citrine fusion construct, *pro*MpDYRK_P:MpDYRK_P-Citrine was constructed as previously reported (Ishizaki et al. 2015). The constructed entry plasmid pENTR1A-*pro*MpDYRK_P:MpDYRK_P ORF, which did not have a stop codon, was used for LR cloning with the destination vector pMpGWB307 (Ishizaki et al. 2015).

The thallus transformation with the CRISPR/Cas9 system and complementation constructs were described previously (Kubota et al. 2013). Thalli from Mpdyrkp-1^{8e} and *Agrobacterium* containing the pMpGWB301-*pro*MpDYRK_P:MpDYRK_P vector or the pMpGWB307-*pro*MpDYRK_P:MpDYRK_P-Citrine vector were used for the transformation. Transformants were selected on half-strength B5 agar medium containing 1 % (w/v) agar, 0.5

μM chlorosulfuron, and 100 mg L⁻¹ cefotaxime (Claforan; Sanofi-Aventis).

Phylogenetic analysis

A phylogenetic tree for the DYRKs was constructed by comparing the amino acid sequences of their kinase domains. The tree was generated using MEGA X (version 10.1, <https://www.megasoftware.net/>; Kumar et al. 2018) with the maximum likelihood algorithm.

Confocal microscopy

For the observation of apical notches, 3-day-old plants after gemma germination were fixed in PFA fixative solution [4% (w/v) paraformaldehyde, and 0.05% (v/v) Triton X-100 in Phosphate buffered saline (PBS)]. Samples were degassed by vacuum infiltration and incubated for 1 h at room temperature with gentle shaking. Fixed samples were rinsed with 0.05% (v/v) Triton X-100 in PBS and cleared in 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. Samples were mounted on glass slides with the ClearSee solution for microscopic observations. SCRI Renaissance 2200 fluorescence was visualized using the confocal laser scanning microscope (Olympus FLUOVIEW FV1000, Tokyo, Japan) at excitation and detection wavelengths of 405 and 425–460 nm, respectively. Images of plant tissues expressing the MpDYRK_P-Citrine fusion protein were captured using a Leica TCS SP8 microscope (Leica Microsystems). A 488-nm argon laser was used for excitation, and fluorescence detected between 500 and 550 nm and 705–765 nm was from Citrine and chlorophyll, respectively.

5-Ethynyl-2'-deoxyuridine (EdU) staining

To visualize S-phase cells, the Click-iT EdU Imaging Kit (Life Technologies) was used. EdU staining was performed as described previously (Furuya et al. 2018; Naramoto et al. 2019; Nishihama et al. 2015), with a slight modification. Three-day-old plants after gemma germination were soaked in half-strength Gamborg's B5 medium with 10 μM EdU at 22 °C for 1 h under continuous white light. Thalli were fixed in FAA [50 % (v/v) ethyl alcohol, 2.5 % (w/v) glacial acetic acid, and 2.5 % (w/v) formalin] 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, samples were treated with the ClearSee solution for a few days. Samples were mounted on glass slides with the ClearSee solution; Alexa Fluor555

fluorescence was visualized using the confocal laser scanning microscope (Olympus FLUOVIEW FV1000, Tokyo, Japan) at excitation and detection wavelengths of 559 and 570–670 nm, respectively. Z-projection images were created using ImageJ software.

Scanning electron microscopy

Thalli fixed in FAA were dehydrated using a graded ethanol series and isoamyl acetate, and dried using a JCPD-5 critical point dryer (JEOL, Tokyo, Japan). The samples were mounted using carbon tape and coated with platinum using a JEC-3000FC sputter-coater (JEOL). Images of epidermal cell surfaces were captured using a JSM-6510LV scanning electron microscope (JEOL, Tokyo, Japan). Epidermal cell margins were traced using Photoshop software (Adobe) and analyzed using ImageJ software. For observation of antheridiophores, samples were frozen in liquid nitrogen and captured using a VHX-D500 microscope (KEYENCE, Osaka, Japan).

Results

Liverwort *M. polymorpha* has a single DYRK ortholog

The plant-specific subgroup in the DYRK family, known as the DYRK subgroup, widely exists from algae to seed plants (Kajikawa et al. 2015; Schulz-Raffelt et al. 2016). Based on a BLAST search and phylogenetic analysis, we identified a single member in the DYRK subgroup, MpDYRK (Mp3g19940), in the genome of the basal land plant *M. polymorpha* (MarpolBase genome database [v5.1: <http://marchantia.info>]; Fig. 1; Montgomery et al. 2020). MpDYRK has a highly conserved S/T protein kinase domain in the C-terminal region, which is homologous to other DYRK orthologs (Fig. 2a). MpDYRK possesses motifs and amino acids that are important for kinase activity, such as a catalytic loop and a lysine residue, which is a phosphate-anchor to support ATP interactions (Fig. 2b; Aranda et al. 2011). However, although the YxY sequence motif is conserved in the activation loop of the DYRK, DYRK2, and Yak1 subgroups, in the DYRK subgroup it is conserved as C/SxY (Fig. 2b). Transcriptomic analysis of the *M. polymorpha* genome estimated five alternative splicing variants as MpDYRK gene models (Fig. 3a; Bowman et al. 2017; Montgomery et al. 2020). All alternative splicing variants of MpDYRK differed within the N-terminal region (Fig. 3a). The N-terminal region of MpDYRK has no known domains or motifs, as judged by Pfam (<http://pfam.xfam.org>). In contrast, the identification of *de novo* sequence motifs by MEME suites (<https://meme-suite.org/>)

[meme/index.html](https://meme-suite.org/meme/index.html)) revealed that there are highly conserved N-terminal region sequence motifs among the DYRK subgroup (Fig. 2a).

Morphology of the genome-edited Mpdyrkp mutants

To investigate the roles of MpDYRK in *M. polymorpha*, Mpdyrkp genome-edited lines were created using the CRISPR/Cas9 system (Sugano et al. 2018). We designed two target sites to obtain independent alleles for deficient mutants, and consequently isolated two genome-edited lines, Mpdyrkp-1^{se} and Mpdyrkp-2^{se} (Fig. 3b). Mpdyrkp-1^{se}, which is constructed using gRNA1, has a 41-bp insertion, while Mpdyrkp-2^{se}, which is constructed using gRNA2, has a 5-bp substitution and an 11-bp deletion (Fig. 3b).

In 12-day-old plants after gemma germination, both genome-edited lines Mpdyrkp-1^{se} and Mpdyrkp-2^{se} exhibited growth defects with abnormal thallus morphologies when compared with the wild-type line Tak-1 (Fig. 4). The thalli of both Mpdyrkp mutants were shrunken and less flattened, and their margins were waved (Fig. 4). To confirm the contributions of MpDYRK to the morphological features of the Mpdyrkp mutants, we constructed a complementation line, *proMpDYRK:MpDYRK-1/Mpdyrkp-1^{se}*. The thallus morphology of this complementation line was restored to that of the wild-type, demonstrating that the abnormal morphologies of the Mpdyrkp gene-edited lines were caused by the loss-of-function of MpDYRK (Fig. 4). This complementation line uses a 5 kbp region upstream of the start codon as an estimated promoter region and its coding sequence. This promoter region was sufficient to express MpDYRK as a morphological regulator (Fig. 4). To examine in detail the differences between the wild-type and mutant lines, we traced the development of gemmalings for 12 days (Fig. 5). After day 3, both Mpdyrkp mutants showed vertical growth in their thallus margin when compared with the wild-type and complementation lines (Fig. 5), indicating that the morphological defects in Mpdyrkp are expressed in the early developmental stages of the thallus. *M. polymorpha* propagates asexually via gemmae formation (Kato et al. 2020; Shimamura 2016). Gemmae are formed at the bottom of the gemma cup, which is a cup-like organ that develops on the middle rib of the thallus. The shape of the gemma cup in Mpdyrkp-1^{se} was shallow and distorted when compared to the wild-type (Fig. 4). In addition to these morphologies during the vegetative growth phase, morphological differences in the reproductive organs were observed. The male reproductive organs, antheridiophores, of Mpdyrkp-1^{se} and Mpdyrkp-2^{se} showed abnormal morphology with less flattening and waved margins, similar to its thallus morphology (Fig. 6). In addition, the stalks of antheridiophore

Fig. 1 Phylogenetic tree of dual-specificity tyrosine phosphorylation-regulated kinases (DYRKs). This phylogenetic tree was constructed by comparing the amino acid sequences of the kinase domains from different plant species (*Arabidopsis thaliana* [At], *Oryza sativa* [Os], *Amborella trichopoda* [Atr], *Selaginella moellendorffii* [Sm], *Marchantia polymorpha* [Mp], *Chara braunii* [Cb], *Klebsormidium nitens* [Kn], and *Chlamydomonas reinhardtii* [Cr]), animals (*Caenorhabdi-*

tis elegans [Ce] and *Homo sapiens* [Hs]), and yeast (*Saccharomyces cerevisiae* [Sc]). Plant DYRKs are indicated with different colors for each plant species. The plant specific DYRK subgroup node is green. The tree was generated using MEGA X with the maximum likelihood algorithm. Bootstrap values (1,000 replicates) are indicated at branch nodes, and the scale bar indicates the number of amino acid substitutions per site

of *Mpdyrkp-1^{se}* and *Mpdyrkp-2^{se}* were thicker than Tak-1 and *proMpDYRKp:MpDYRKp-1/Mpdyrkp-1^{se}* (Fig. 6). These results suggest that *MpDYRKp* plays an important role in tissue morphogenesis in both the vegetative and

reproductive phases. On the other hand, antheridia showed normal development in *Mpdyrkp-1^{se}* and *Mpdyrkp-2^{se}* (Fig. 6i–l).

Fig. 2 DYRK amino acid sequence comparison amongst different species. **a** Sequence motif among DYRKs. Comparison analysis of DYRKs in *A. thaliana* (At), *M. polymorpha* (Mp), *C. elegans* (Ce), and *H. sapiens* (Hs) was performed using MEME suites. **b** Multiple sequence alignments were constructed using MUSCLE. Align-

ments for a part of the kinase domain of DYRKs (AtDYRKs, MpDYRK, CrDYRKs), YAK1s (AtYak1 and MpTAR1), and DYRK2 (MpDYRK2) are indicated. Residues were colored according to polarity. Important sequence features for kinase activity are indicated

Fig. 3 Construction of *Mpdyrkp* genome-edited lines. **a** Genomic structure of the encoding region for the *MpDYRKP* gene. Red bars indicate the locations of the target sites for the gRNAs. **b** Mutations detected by sequencing analysis. The guide RNA including the PAM

sequence (red) and a part of the *MpDYRKP* sequence in both the wild-type and *Mpdyrkp^{ge}* lines are shown. The genome-edited line *Mpdyrkp-1^{ge}* has a 41-bp insertion, while *Mpdyrkp-2^{ge}* has a 5-bp substitution and a 11-bp deletion (orange)

Subcellular localization pattern of *MpDYRKP*

To analyze the gene expression pattern of *MpDYRKP*, we re-analyzed the published RNA-seq data sets from various tissues containing thallus, reproductive tissues (antheridiophore, antheridium, and archegoniophore), and developing spores (Bowman et al. 2017; Higo et al. 2016). *MpDYRKP* was broadly expressed in all the analyzed tissues (Fig. S1). For detailed expression pattern and protein localization analysis, we constructed a reporter line, *proMpDYRKP:MpDYRKP-Citrine-1/Mpdyrkp-1^{ge}*, which used a 5 kbp region upstream from the start codon of *MpDYRKP* as a promoter, a process similar to that used for the complementation line. Our observations of this reporter line revealed that *MpDYRKP-Citrine* was strongly detected around the apical notch (Fig. 7). Moreover, at the subcellular level, *MpDYRKP-Citrine* was observed as a diffuse punctate structure in the cytosol (Fig. 7). This subcellular localization pattern is different from that of *A. thaliana* DYRKP-2 A, which is uniformly located in the cytosol with no punctate structure (Iwabuchi et al. 2019).

Cell proliferation activity of *Mpdyrkp* mutants

During thallus development, cell proliferation mainly occurs in the apical notches (Shimamura 2016). Thus, the apical notch is important for thallus morphogenesis. Although the *Mpdyrkp* gene-edited lines exhibited abnormal

thallus morphology (Figs. 4 and 5), presense of the apical or subapical cells in the apical notch region were recognized in both *Mpdyrkp-1^{ge}* and *Mpdyrkp-2^{ge}* as well as Tak-1 and *proMpDYRKP:MpDYRKP-1/Mpdyrkp-1^{ge}* (Fig. 8a–d). Next, we measured the number of S-phase cells visualized by EdU staining in the apical notches of 3-day-old plants to assess the cell proliferation activity. The number of EdU signals in the apical notches of *Mpdyrkp-1^{ge}* was comparable to that of Tak-1 and *proMpDYRKP:MpDYRKP-1/Mpdyrkp-1^{ge}* (Fig. 8e–i). These results suggest that both apical notch formation and cell proliferation activity are not casual factors for the morphological abnormalities in the *Mpdyrkp* mutants. On the other hand, *Mpdyrkp-2^{ge}* exhibited smaller number of EdU signals in their apical notches compared with Tak-1 (Fig. 8e–i), suggesting that *Mpdyrkp-2^{ge}* may have additional mutations relate to the cell proliferation activity.

Epidermal cell morphology of *Mpdyrkp* mutants

The morphological defects in the *Mpdyrkp* genome-edited lines, suggests that *MpDYRKP* acts as a morphological regulator (Figs. 4 and 5). As tissue morphogenesis is an accumulation of cellular morphogenesis, we observed the genome-edited lines, *Mpdyrkp-1^{ge}* and *Mpdyrkp-2^{ge}*, using scanning electron microscopy. In contrast to the smooth epidermal surface of Tak-1, the epidermal surfaces of both *Mpdyrkp-1^{ge}* and *Mpdyrkp-2^{ge}* appeared uneven and

Fig. 4 Morphology of *Mpdyrpk* mutants. **a–d** 12-day-old gemmalings of the wild-type line Tak-1 (**a**); the *Mpdyrpk* genome-edited lines *Mpdyrpk-1^{ge}* (**b**) and *Mpdyrpk-2^{ge}* (**c**); and the complementation line *proMpDYRKP:MpDYRKP-1/Mpdyrpk-1^{ge}* (**d**). **e–f** Gemma cup of Tak-1 (**e**) and *Mpdyrpk-1^{ge}* (**f**) cultured for 16 days. Scale bars: 2 mm (**a–d**), 1 mm (**e, f**)

disordered (Fig. 9a–h). In addition, the shape and size of the air pores in these genome-edited lines were uneven (Fig. 9a–h). Similar trends were observed in the surfaces of antheridial receptacles of both *Mpdyrpk-1^{ge}* and *Mpdyrpk-2^{ge}* (Fig. 9m–t). Moreover, we found that both *Mpdyrpk-1^{ge}* and *Mpdyrpk-2^{ge}* exhibited a tendency for lower circularity in the epidermal cells of thalli than Tak-1 and the complementation line (Fig. 9i–l). These results suggest that MpDYRKP contributes to thallus morphogenesis, at least in part, via the regulation of epidermal cell shape.

Discussion

MpDYRKP is involved in the regulation of tissue morphogenesis

Mpdyrpk genome-edited lines of the model basal land plant *M. polymorpha*, were shown here to exhibit morphological defects in tissue morphogenesis in both the vegetative and reproductive phases (Figs. 4, 5 and 6). This is the first report regarding the phenotype of a loss-of-function mutant of a DYRKP ortholog in bryophytes. At the cellular level, the epidermal cells were found to have an altered shape with lower circularity in the *Mpdyrpk* mutants when compared with the wild type (Fig. 9). According to abnormal cell shapes, the epidermal surface of the *Mpdyrpk* mutants was uneven and disordered (Fig. 9). The disorder in the shape and alignment of the epidermal cells of the *Mpdyrpk* mutants may account for the morphological disruption of orderly tissues such as the thalli, air pores, and gemma cups (Figs. 4 and 9). These results suggest that MpDYRKP regulates cell

Fig. 5 Time-course observation of *Mpdyrpk* mutants. The wild-type line Tak-1; the *Mpdyrpk* genome-edited lines *Mpdyrpk-1^{ge}* and *Mpdyrpk-2^{ge}*; and the complementation line;

proMpDYRKP:MpDYRKP-1/Mpdyrpk-1^{ge} were observed at the indicated time points during growth from the gemma. Scale bars: 2 mm

Fig. 6 Morphology of the male reproductive organs of *Mpdyrpk* mutants. Side (a–d) and top (e–h) views of antheridiophores of Tak-1 (a, e), *Mpdyrpk-1^{se}* (b, f), *Mpdyrpk-2^{se}* (c, g), and *proMpDYRKP:MpDYRKP-1/Mpdyrpk-1^{se}* (d, h). Antheridia of Tak-1 (i), *Mpdyrpk-1^{se}* (j), *Mpdyrpk-2^{se}* (k), and *proMpDYRKP:MpDYRKP-1/Mpdyrpk-1^{se}* (l). Scale bars: 1 cm (a–h), 100 μm (i–l)

morphology to enable well-ordered development in *M. polymorpha*. Generally, cytoskeletons, including microtubules and actin filaments, play important roles in the regulation and determination of plant cell shape (Smith and Oppenheimer 2005). It has been suggested that *A. thaliana* DYRKPs (AtDYRKPs) are involved in nuclear positioning via the regulation of actin filament alignment (Iwabuchi et al. 2019). Moreover, in humans, a member of the DYRK1 subgroup, DYRK1A, induced the destabilization of microtubules via the hyperphosphorylation of tau proteins, which is a causal

Fig. 7 Subcellular localization of MpDYRKP-Citrine protein. a and b Subcellular localization of MpDYRKP-Citrine in 1-day-old gemmalings of *proMpDYRKP:MpDYRKP-Citrine-1/Mpdyrpk-1^{se}* transgenic plant was visualized with DIC image Citrine signals are shown in green and autofluorescence in magenta. b Is a magnified image from around the apical notch in a. Scale bars: 50 μm

agent of Alzheimer's disease (Ryoo et al. 2007; Woods et al. 2001). Thus, it is important to analyze the molecular functions and identify the interacting proteins of MpDYRKP to understand the underlying mechanisms of tissue morphogenesis in *M. polymorpha*.

Relationship between DYRKPs and AN

In *A. thaliana*, DYRKPs were found to interact with AN proteins in the yeast two-hybrid screens (Bhasin and Hülskamp 2017: ANGSTIFOLIA INTERACTING KINASE [AIK1] corresponds to AtDYRKP-1), and the co-immunoprecipitation assay using transgenic plants expressing AN-GFP (Iwabuchi et al. 2019). Mutants deficient in AN exhibited multiple morphological phenotypes, such as narrow and thicker leaves, twisted fruits and petals, and the premature opening of flowers (Bai et al. 2010, 2013; Rédei 1962; Tsuge et al. 1996; Tsukaya et al. 1994). In *M. polymorpha*, the knockout mutants of the AN ortholog, *Mpan*, showed abnormal twisted thalli and suppressed thallus growth, indicating that MpAN is also involved in the regulation of tissue morphogenesis (Furuya et al. 2018). Moreover, in both *A. thaliana* and *M. polymorpha*, the loss of function of AN results in an abnormal arrangement of the cortical microtubules (Furuya et al. 2018; Kim et al. 2002). The phenotypes between *Mpdyrpk* and *Mpan* mutants were partially shared in the context of abnormal morphologies with less flattening of the thalli and antheridial receptacles (Figs. 4, 5 and 6). However, it is not easy to compare the phenotypic similarities between the *Mpdyrpk* and *Mpan* mutants due to their morphological complexity. In terms of epidermal cell shapes, *Mpdyrpk* showed lower circularity than the wild type under normal growth conditions (Fig. 9), whereas *Mpan* showed higher circularity only under weak blue light conditions (Furuya et al. 2018). These results indicate that the

Fig. 8 Cell proliferation activity in *Mpdyrpk* mutants. **a–d** Confocal images of the apical notches in the Tak-1 (**a**), *Mpdyrpk-1^{ge}* (**b**), *Mpdyrpk-2^{ge}* (**c**), and *proMpDYRKP:MpDYRKP-1/Mpdyrpk-1^{ge}* (**d**). Plants cultured for 3 days after gemma germination were treated with ClearSee solution. Cell walls stained by SCRI Renaissance 2200 are shown in white. Red dots indicate apical and/or subapical cells. **e–h** S-phase nuclei visualized by EdU in the Tak-1 (**e**), *Mpdyrpk-1^{ge}* (**f**), *Mpdyrpk-2^{ge}* (**g**), and *proMpDYRKP:MpDYRKP-1/Mpdyrpk-1^{ge}* (**h**). Plants cultured for 3 days after gemma germination were treated with EdU-containing liquid medium for 1 h. Z-series fluorescence images

were obtained. Maximum projection images are shown. EdU-containing nuclei are shown in red. **i** The numbers of EdU-containing nuclei 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 $\times 1.5$ the interquartile ranges. The black dot indicates an outlier. Gray dots indicate each sample ($n = 10$ apical notches). Different lowercase letters indicate significant differences ($P < 0.05$; Tukey–Kramer test). Scale bars: 100 μm (**a–d**), 50 μm (**e–h**)

effects of the *Mpdyrpk* mutation are more severe and opposing to those of the *Mpan* mutation. The protein localization of MpDYRKP-Citrine was observed as a diffuse punctate structure in the cytosol (Fig. 7). This localization pattern is different from that of GFP-DYRKP-2 A in *A. thaliana*, which is uniformly located in the cytosol with no punctate structure (Iwabuchi et al. 2019). Interestingly, the diffuse punctate structures observed in MpDYRKP-Citrine transgenic plants were similar to the AN protein localization pattern observed in both *M. polymorpha* and *A. thaliana* (Bhasin and Hülskamp 2017; Furuya et al. 2018). Further studies of the molecular and genetic interactions between

DYRKP and AN in *M. polymorpha* will provide clues to uncover their functional mechanisms in tissue and cell morphogenesis.

Conclusions

In this study, we revealed that MpDYRKP is involved in the regulation of cell shape to enable the formation of orderly tissue morphologies in *M. polymorpha*. However, the molecular function, target substrates, and physiological roles of MpDYRKP and other DYRKPs remain unknown.

Fig. 9 Epidermal morphology in *Mpdyrpk* mutants. **a–h** Scanning electron microscopy images of thalli of Tak-1 (**a, e**), *Mpdyrpk-1^{ge}* (**b, f**), *Mpdyrpk-2^{ge}* (**c, g**), and *proMpDYRKP:MpDYRKP-1/dyrkp-1^{ge}* (**d, h**). **i–l** Epidermal-cell circularity shown in scanning electron microscopy images (**e–h**) were measured using Image J. **m–t**

Scanning electron microscopy images of antheridial receptacles of Tak-1 (**m** and **q**), *Mpdyrpk-1^{ge}* (**n** and **r**), *Mpdyrpk-2^{ge}* (**o, s**), and *proMpDYRKP:MpDYRKP-1/dyrkp-1^{ge}* (**p, t**). Scale bars: 1 mm (**a–d, m–p**), 100 μm (**e–h, q–t**)

Comparison of the DYRKPs revealed that features of the amino acid sequences of MpDYRKP are conserved across a broad range of plant species. Thus, the molecular functions of MpDYRKP, as a morphological regulator in *M. polymorpha*, may have been conserved during plant evolution.

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