KANADI promotes thallus differentiation and FR-induced gametangiophore formation in the liverwort Marchantia

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Summary

- In angiosperms, KANADI transcription factors have roles in the sporophyte generation regulating tissue polarity, organogenesis and shade avoidance responses, but are not required during the gametophyte generation. Whether these roles are conserved in the gametophyte-dominant bryophyte lineages is unknown, which we examined by characterising the sole KANADI ortholog, MpKAN, in the liverwort Marchantia polymorpha.
- In contrast to angiosperm orthologs, MpKAN functions in the gametophyte generation in Marchantia, where it regulates apical branching and tissue differentiation, but does not influence tissue polarity in either generation. MpKAN can partially rescue the sporophyte polarity defects of kanadi mutants in Arabidopsis, indicating that MpKAN has conserved biochemical activity to its angiosperm counterparts.
- Mpkan loss-of-function plants display defects in far-red (FR) light responses. Mpkan plants have reduced FR-induced growth tropisms, have a delayed transition to sexual reproduction and fail to correctly form gametangiophores.
- Our results indicate that MpKAN is a modulator of FR responses, which may reflect a conserved role for KANADI across land plants. Under FR, MpKAN negatively regulates MpDELLA expression, suggesting that MpKAN and MpDELLA act in a pathway regulating FR responses, placing MpKAN in a gene regulatory network exhibiting similarities with those of angiosperms.

Introduction

The evolution of land plants from an ancestral alga more than 450 million years ago was facilitated by changes in life history, body plan and responses to new environmental cues on land. Recent analyses of streptophyte genomes have indicated key genetic innovations, which helped facilitate the transition to land, with a small number of transcription factor (TF) families and phytohormone pathways either originating or displaying increased diversity in the ancestral land plant (Hori et al., 2014; Ju et al., 2015; Catarino et al., 2016; Bowman et al., 2017; Cheng et al., 2019; Li et al., 2020). The elucidation of ancestral vs derived functions provides insight into the nature of the ancestral land plant and how genes direct development of plants with radically different body plans.

Among the land plant-specific TFs are the KANADI (KAN) TFs, a subfamily of GARP (GOLDEN2, Arabidopsis response regulator (ARR) and Phosphorus Stress Response1 (PSR1)) transcriptional repressors, which possess conserved GARP DNA-binding and EAR motif repressor-like domains (PDLSL and LEFTL), with the latter domains likely to facilitate interactions with TOPELESS co-repressors (Hosoda et al., 2002; Caussier et al., 2012; Bowman et al., 2017; Safi et al., 2017). In Arabidopsis, KAN is not required for gametophyte development (Izhaki & Bowman, 2007), but functions in the sporophyte generation, where it regulates tissue polarity by specifying abaxial–peripheral tissue types in leaves and vasculature, as well as organogenesis by co-ordinating lateral organ initiation sites (Eshed et al., 2001, 2004; Kerstetter et al., 2001; Emery et al., 2003; Heisler et al., 2005; Caggiano et al., 2017; Ram et al., 2020). Additionally, KAN TFs have been shown to regulate hypocotyl elongation and vasculature responses to shade (Brandt et al., 2012; Xie et al., 2015; Merelo et al., 2017; Botterweg-Paredes et al., 2020). A model has been proposed whereby KAN and Class III HOXDOMAIN-LEUCINE ZIPPER (C3HDZ) TFs regulate both development and shade processes by the antagonistic regulation of shared target genes, including genes encoding auxin biosynthesis enzymes (i.e. TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA) and YUCCA (YUC)) and Class II HD-ZIP (C2HDZ) TFs (Brandt et al., 2012; Merelo et al., 2013, 2017; Reinhart et al., 2013; Huang et al., 2014; Xie et al., 2015). Thus, distinct processes (developmental patterning and shade avoidance) are regulated by a shared KAN-C3HDZ regulatory module (Merelo et al., 2017). During shade avoidance responses, auxin biosynthesis genes are also downstream targets of the PHYTOCHRONE-
INTERACTING FACTOR (PIF) TFs, and shade-induced elongation growth is associated with high auxin levels, thus providing a potential link between KAN-C3HDZ regulatory activity and phytochrome-mediated regulation of shade avoidance responses (Tao et al., 2008; Hornitschek et al., 2012; Li et al., 2012; Merelo et al., 2017).

The role of KAN TFs in nonangiosperm lineages, however, is not well understood. Expression analysis of vascular plant KAN orthologs suggests that a role in regulating tissue polarity may be conserved in other eufyllophytes (ferns and seed plants) (Zumajo-Cardona et al., 2019). The function of KAN in the bryophyte lineages (i.e. liverworts, hornworts and mosses) remains unknown. In contrast to the four KANADI homologs present in Arabidopsis, Marchantia has a single ortholog - MpKAN – which by parsimony reflects the ancestral copy number (Bowman et al., 2017). To better understand the ancestral role of KAN in land plants, we characterised the sole Marchantia ortholog – MpKAN – in both generations of the life cycle.

Materials and Methods

MpKAN promoter cloning

Marchantia gene nomenclature follows the guidelines designed by Bowman et al. (2016). Gene and coding nucleotide sequences of MpKAN (v.6.1: Mp3g04970.1) were collected from the MarpolBase genome portal (Supporting Information Fig. S1). All primers are listed in Table S1. Primer sets #1/#2 and #3/#4 were used to amplify 6.1kb of MpKAN coding sequence cloning

Wild-type cDNA from thallus tissue was used to amplify a 2.1-kb MpKAN coding sequence (CDS) with and without a stop codon (primers #9–#11), and cloned into pENTR/D-TOPO (Life Technologies). To generate a T-DNA for the complementation of MpKAN-CDS population was produced, and Tissues from three independent lines were isolated (Fig. S2).

Complementation of MpKAN lines

Complementation of MpKAN plants was attained by first crossing Mpkan-5ge male and wild-type female lines to generate Mpkan-5ge/+ sporangia. Sporelings produced from Mpkan-5ge/+ sporangia were transformed with pMpKAN:Ca9-MpKAN-CDS-pMpGW401 T-DNA. In this manner, a 1 : 1 Mpkan-5ge/+pMpKAN:Ca9-MpKAN-CDS population was produced, and complemented Mpkan-5ge × MpKAN:Ca9-MpKAN-CDS lines were identified by genotyping using primer sets #12/#13 and #14/#15.

GUS staining

Tissues from three independent pMpKAN:GUS lines were incubated in GUS staining solution (1mM potassium ferrocyanide, 1mM potassium ferricyanide and 1mM X-Gluc) for 2–4 h at 37°C and cleared with 70% ethanol.

Growth conditions

Plant growth conditions were presented as outlined in previous studies (Gamborg et al., 1968; Ishizaki et al., 2008; Flores-Sandoval et al., 2015). For controls, Australia/Melbourne accession wild-type male and female lines were used. Plants were grown under either constant white light (WL, R : FR = 6) or FR-enriched white light (FR, R : FR = 0.5), with spectral compositions identical for both light treatments apart from FR light. For experiments using the auxin analog 2,4-dichlorophenoxyacetic acid (2,4-D), plants were grown on ½ B5 media supplemented with mock (equivalent vol. 100% EtOH) or 0.5 μM 2,4-D. For pMpKAN:GR experiments, plants were grown on ½ B5 media supplemented with either mock (equivalent vol. 100% EtOH), 1 μM DEX or 10 μM DEX.

Plant transformation and generation of loss-of-function alleles

All transgenic lines were generated by the transformation of Australia/Melbourne accession wild-type sporelings, unless otherwise specified. Marchantia sporelings were transformed following Ishizaki et al. (2008). To generate CRISPR/Cas9-mediated Mpkan mutants, sporelings were cotransformed with MpKAN-gRNA1-pMpGE010 and MpKAN-gRNA2-pMpGW301. Lines harbouring loss-of-function alleles were genotyped by sequencing PCR amplicons using primers #12/#13. Two independent lines – Mpkan-5ge (a male line) and Mpkan-7ge (a female line) – were isolated (Fig. S2).
Tissue sectioning, in situ hybridisation and scanning electron microscopy

Tissue sectioning and in situ hybridisation were performed as described previously (Zachgo, 2002; Retamales & Scharaschkin, 2014). Histological sections were stained with either toluidine blue (1 min) for thallus sections or toluidine blue (1 min) followed by ruthenium red (1 min) for sporophyte sections. The sections were set to 7 and 8 μm thicknesses for histological and in situ sections, respectively. In situ hybridisation was performed using hydrolysed (200 bp) digoxigenin-labelled antisense MpKAN mRNA as probe. Tissue fixation and SEM were performed as described previously (Flores-Sandoval et al., 2015).

Phenotype quantification

Air pore counts, thallus area measurements and quantification of dorsal differentiation indices were measured with 7-d gemmational dorsal differentiation indices were measured with 7-d gemmulation and substrate (Efroni et al., 2008). The calculation of the angle between the thallus and substrate (θTS), see Methods S1.

Calculation of θTS

For the methodology of measuring the angle between the thallus and substrate (θTS), see Methods S1.

RNAseq experiments

To identify transcriptional targets of MpKAN, RNA sequencing (RNA-seq) was used to compare the transcript abundance between wild-type, Mpkaban and proMpEFI: MpKAN-GR plants. Transcriptome data were collected from wild-type and Mpkaban lines grown under continuous white light (WL = 15 d WL) and FR-enriched light (FR = 10 d WL, then 5 d FR) (Methods S1). For transcriptomes generated from wild-type male and proMpEFI: MpKAN-GR L1 male plants, RNA was extracted from 15-d germalens subsequently treated with either (1) mock (0 h), (2) 10 μM dexamethasone (DEX) (3 h) or (3) 10 μM DEX (24 h) (Methods S1).

Whole thallus material was collected from three replicates for each independent line. Total RNA was isolated (RNAeasy Plant Kit, Qiagen, Germany) and assessed for quality using a Nanodrop spectrophotometer and a 2100 Bioanalyzer (Agilent, Waldbronn, Germany). Library preparation used polyA mRNA selection and MGIEasy stranded mRNA chemistry. Sequencing used MGI Tech MGISEQ-2000RS hardware (400 million raw reads per lane, 100-pb paired-end reads). Raw reads were mapped onto the Marchantia polymorpha v.5.1 assembly using the HISAT2 alignment software (Kim et al., 2015). Read counting was performed using FEATURECounts to generate matrices of raw reads per gene locus (Liao et al., 2014). Raw counts and normalised transcripts per million (TPM) values for each loci (Table S2).

To corroborate the similarity between replicates for each genotype and condition, principal component analysis (PCA) was performed using R v.3.6.1 and the DEseq2 function (S3), with raw reads normalised as log-transformed TPM values. Pairwise differential gene expression analysis was performed using the edgeR software package (Robinson et al., 2010). Raw outputs of each edgeR pairwise comparison are included in Table S2. Only genes with associated adjusted (adj.) P values of < 0.001 were considered differentially expressed.

Volcano plots were generated using the ggplot2 function in R v.3.6.1 and were annotated for genes encoding transcription regulators, phytohormone-related proteins, peptide/receptors or light signalling proteins (Table S3). Genes were considered downregulated in DEX-treated proMpEFI: MpKAN-GR plants when the following conditions were met: (1) logFC < −1, MpKAN-GR 3 h DEX/WT 3 h DEX; (2) logFC < −1, MpKAN-GR 24 h DEX/WT 24 h DEX; and (3) logFC ≥ 0, WT 3 h DEX/WT 0 h mock. Genes were considered up- or downregulated in Mpkaban plants relative to wild type when logFC values were > 1 or < −1, respectively, for pairwise comparisons between genotypes and independent of light treatment. For the analysis of differentially expressed genes (DEGs) in Mpkaban and wild-type plants under FR compared with WL, genes were considered FR-upregulated when logFC > 1 and FR-downregulated when logFC < −1 for pairwise comparisons between light treatments and within genotypes. To generate a robust list of gene candidates negatively regulated by MpKAN, genes with logFC > 0.5 in all Mpkaban/WT pairwise comparisons and logFC < −0.5 in MpKAN-GR 3 h DEX/WT 3 h DEX and MpKAN-GR 24 h DEX/WT 24 h DEX pairwise comparisons were selected. To remove genes that were additionally downregulated in DEX-treated wild-type plants, genes were also required to have logFC ≥ 0 for the WT 3 h DEX/WT 0 h mock pairwise comparison. GO enrichment analysis was performed on selected gene lists using Plant Transcriptional Regulatory Map (http://plantregmap.cbi.pu.edu.cn/go.php; Peking University) and using Fisher’s exact test to identify statistically significantly enriched GO terms. TPM plots for selected gene loci of interest were generated using the PlotsOfData web application (Postma & Goedhart, 2019).

Arabidopsis complementation experiments

For proAtKAN1: MpKAN kan1-2 kan2-1 rescue experiments in Arabidopsis, proAtKAN1: MpKAN was created by cloning the MpKAN CDS downstream of the AtKAN1 (At5g16560) promoter that consists of a 884-bp fragment of the conserved second intron fused to a 5.3-kb fragment upstream of AtKAN1, which has been previously described (Efroni et al., 2008). The proAtKAN1: MpKAN construct was then subcloned into pMLBART. Transgenic lines were generated by Agrobacterium-mediated transformation into wild-type Columbia and kan1-2 kan2-1 plants. Plants were grown under long-day conditions (16-h day), and transformants were selected on soil for BASTA resistance. Presumptive proAtKAN1: MpKAN kan1-2 kan2-1 T1 plants were sequenced to determine their genotype. The region
of the first exon corresponding to the respective kan1-2 and kan2-1 mutations was PCR-amplified and PCR-sequenced to confirm the homozygosity of mutant alleles (Eshed et al., 2001) (primers #16–#19).

**Results**

**Expression of MpKAN in gametophyte vegetative and reproductive tissues**

To ascertain the gametophyte spatiotemporal expression pattern of MpKAN in Marchantia, _proMpKAN:GUS_ transcriptional reporter lines including 6.1 kb upstream of the _MpKAN_ translation start site were stained each day from gamma germination to form a time series of expression. After removal from the gametemata cups (i.e. 0 d), gametemata exhibited staining localised to the apical notches, where the apical meristems are located, with reporter gene expression maintained at the apical notches following germination (Fig. S4a–e). _In situ_ hybridisation on longitudinal thallus sections also indicated _MpKAN_ expression in the region of the apical notch, in both dorsal and ventral epidermal and subepidermal cell files (Fig. 1a). At the commencement of the first bifurcation event after 4 d, reporter gene expression was detected at both apical notches, as well as in photosynthetic filaments of developing air chambers (Fig. 1b). In addition to staining in the apical notches, adult thalli exhibited staining along the midrib of the thallus, and at the bases of primordial gametemata cups, and _in situ_ hybridisation additionally indicated the expression of _MpKAN_ in developing gametemata within gametemata cups (Fig. S4f–h). To determine whether _MpKAN_ expression differed in plants grown under FR, as experienced in shade, gemmalings were also grown under WL for 18 d, followed by FR for 7 d. The staining pattern in vegetative tissues of _proMpKAN:GUS_ plants was largely unchanged under FR (Fig. S4i). After prolonged FR exposure, thallus transition to sexual reproduction via the production of gemmangia in specialised gametangium-bearing structures (i.e. gametangiophores). GUS staining was additionally observed in gametangiophores during sexual reproduction, primarily in the receptacles which harbour gametangia (Figs 1c,d, S4i,j,k). In the sporophyte generation, _in situ_ hybridisation indicated that _MpKAN_ is expressed broadly in developing sporophytes, with no localisation of expression along the axes of symmetry as would be expected for genes that regulate tissue polarity in the sporophyte generation (Fig. S4l,m).

**MpKAN influences apical growth and differentiation in vegetative tissues**

To investigate _MpKAN_ function, loss-of-function alleles were created using CRISPR-Cas9-mediated mutagenesis, using two gRNAs coexpressively coexpressed in transformants to generate deletion alleles (Fig. 1c). Two independent transgenic lines – _Mpkan-5ge_ (a male) and _Mpkan-7ge_ (a female) – with 3.3-kb deletions of genomic sequence spanning the region between gRNA target sites were identified (Fig. S5a–c). The deletion in both lines included 385 bp of exonic sequence encoding 57% of the GARP DNA-binding domain and caused a frameshift putatively disrupting the LEFTL domain (Fig. 1e). Transcriptome reads demonstrated that _Mpkans_ lines expressed a transcript lacking exons 2–5 (Fig. 1f). To confirm that _Mpkans_ phenotypes described below can be attributed to _Mpkans_ loss-of-function alleles, mutant alleles were complemented. Two independently complemented _Mpkan-5ge_ lines expressing _proMpKAN:MPKAN-CDS_ were corroborated by genotyping (Fig. S5d,e). Complemented _Mpkan-5ge_ lines showed restored wild-type phenotypes (Figs 1g–o, 3c–h) with the exception that gamemata cups were not restored (Fig. 1p). The lack of complete complementation suggests that not all endogenous regulatory elements are present in the complementation construct.

_Mpkan_ mutants were of similar size to wild-type plants, and dorsal air pores and air chambers had a morphology comparable to those of wild type (Figs 1m, 6a,b,e). However, significantly fewer air pores were formed in the mutant background (Figs 1n, 6f). Additionally, the first bifurcation event was further progressed in _Mpkans_ plants, with apical notches more laterally displaced from each other (Fig. 2a–f), indicating that the reduction in air pore numbers was not caused by reduced or delayed apical activity. _Mpkans_ plants also developed abnormal, wing-like outgrowths from the central zone of the gemmaling, a region that is not developmentally active in wild type, and produced more dorsal rhizoids than wild type from the central region (Fig. 2a–f). Ventrally, _Mpkans_ plants developed scales and smooth rhizoids of similar morphology and positioning to those of wild type (Fig. 56c,d). No evidence was found of polarity abnormalities in _Mpkans_ plants, with dorsal and ventral tissue types correctly positioned along the dorsiventral axis (Figs 2a,b, 56c,d), and longitudinal sectioning of 5-d gemmalings corroborated that the loss of _MpKAN_ function did not impair the establishment of a dorsiventral thallus (Fig. S6g,h).

We further characterised the apparent differentiation defect in _Mpkans_ dorsal tissue types. After 8-d growth from gamemata, a distinct boundary is discernible between differentiated dorsal tissue originating from the apical cell, herein referred to as the differentiation zone (dz), and a region derived from the central region of the gemma where cells do not divide and remain developmentally inactive, referred to as the central zone (cz) (Methods S1). To quantify the extent of dorsal differentiation, the dz surface area was measured and calculated as a fraction of total dorsal surface area to give a value called the ‘differentiation area index’. Differentiation area indices were significantly reduced in _Mpkans_ mutants (Fig. 2g), indicating that they are compromised in their ability to differentiate dorsal tissue types.

_Mpkan_ mutants produced more apical notches and fewer gamemata cups when compared with wild type (Figs 1o,p, 2i–l, S6i,j). Since apical notch numbers were increased in _Mpkans_ plants, we assessed whether the phenotype reflected faster apical branching during specific plastochrons. To test this, plastochron duration was measured for each of the first three branching events. Plastochron 1 (the time taken to bifurcate from 2 to 4 notches) was completed in significantly fewer days in _Mpkans_ lines compared with wild type, whereas plastochrons 2 and 3 took a comparable number of days to be
Fig. 1 Expression analysis of MpKAN and generation of Mpkan loss-of-function plants in Marchantia. (a) MpKAN transcripts were detected via in situ hybridisation in the immediate dorsal and ventral derivatives of apical cell divisions in longitudinal sections of thallus apical notches. Asterisk indicates putative site of apical cell. d., dorsal; v, ventral; ap, air pore. (b–d) Expression patterns of MpKAN as assessed by \(\text{mpKAN}:\text{GUS}\) reporter gene expression in (b) 4-d-old gemmalings, (c) archegoniophores and (d) antheridiophores. (b) In 4-d-old gemmalings, when apical notches first bifurcated during plastochron 1, expression was detected in the apical notches (asterisks) and the filaments (arrow) of air chambers closest to the apical notch. (c, d) Reporter gene expression was observed in archegoniophores (c) and antheridiophores (d), predominantly in receptacle tissues. (e) Schematic diagram depicting Mpkan gene structure, domain-encoding sites and gRNA sites for mRNA sequence of wild-type and loss-of-function alleles. Mpkan-5\(^{ge}\) and Mpkan-7\(^{ge}\) harbour deletions at gRNA1 and gRNA2 targeting sites. (f) Raw RNA-seq density reads (Sashimi plots) of wild-type and Mpkan lines at the MpKAN locus. Wild-type MpKAN mRNA consists of six exons, whereas Mpkan alleles resulted in loss of expression of exons 2–5. WT (g–h), Mpkan (i–j) and Mpkan-5\(^{ge}\) × \(\text{pmpKAN}\):MpKAN-CDS (complemented) (k, l) plants grown for 7 d from gemmae. Asterisks indicate apical notches. (m) Wild-type, Mpkan and complemented lines had largely similar thallus areas. Complemented lines showed restored branching and air pore numbers (n, o), but not gemmae cup numbers (p). (m–p) Data are mean ± SD. Statistical differences among genotypes were determined using one-way ANOVA and Tukey’s multiple comparisons tests, with letters indicating statistically significant groups (\(P < 0.01\)). Where present, numbers after genotype indicate independent lines. Bars: (a) 125 \(\mu\)m; (b–d) 100 \(\mu\)m; (e) 500 bp; (g–l) 2 mm.
completed (Fig. 2h). In agreement with this, SEM images of gemmalings showed that Mpkan mutants were already markedly bifurcating at this timepoint, whereas in wild type, bifurcation was either yet to commence or in its early stages (Fig. S6k–n). Altogether, the increase in apical notch number in Mpkan plants was caused primarily by an increased rate of bifurcation during plastochron 1.

Since KAN orthologs in angiosperms are active in the sporophyte generation, and in situ hybridisation indicated expression of MpKAN in the sporophyte, we next examined the phenotypes

![Image of gemmalings showing differences between wild type and Mpkan mutants](https://example.com/image.png)
of Mp kan homozygous sporophytes. Homozygous Mp kan sporophytes were not impaired in polarity along either radial or apical–basal axis of symmetry, with distinct foot, seta and capsule tissues forming in correct positions along the apical–basal axis, and amphithelial (i.e. capsule wall cell types) and endothelial (i.e. spores and elaters) layers correctly positioned along the radial axis of the capsule (Fig. S6o–r). Thus, similarly to gametophyte tissues, we found no evidence, suggesting MpKAN regulates polarity in the sporophyte.

Given that KAN represses auxin biosynthesis gene expression in angiosperms (Brandt et al., 2012; Merelo et al., 2013; Xie et al., 2015), we looked for the presence of auxin-related phenotypes in Mp kan plants. Wild-type gemmalings grown on low concentrations of the auxin analog 2,4-D display two developmental abnormalities. First, numerous rhizoids are produced from the dorsal cz (Fig. S7a,b) (Tarén, 1958; Flores-Sandoval et al., 2015). Second, secondary thalli are occasionally initiated at the cz/dz boundary (Fig. S7a,b), a process thought to be caused by growth from a second gemma apical cell that under normal conditions is repressed via the eventual establishment of a dorsiventral auxin gradient (Halbsguth & Kohlenbach, 1953). Similar to 2,4-D-treated wild-type plants, mock-treated Mp kan plants produce profuse dorsal rhizoids from the cz and occasionally produce secondary thalli from the cz/dz boundary (Fig. S7c–e). Together, these results suggest that Mp kan lines phenocopy pharmacological treatment with low auxin concentrations.

**MpKAN as a modulator of growth responses to FR-enriched light**

Given that KAN TFs regulate FR light responses in angiosperms (Merelo et al., 2017; Botterweg-Paredes et al., 2020), we next examined whether the loss of MpKAN activity affected FR responses in Marchantia. The growth under FR causes two developmental changes compared with WL, namely a transition from plagiotropic to orthotropic growth, with the thallus tips increasingly elevated from the substrate, and the transition to sexual development via the formation of gametangiophores (Fig. S8a–f) (Fredericq & de Greef, 1966; Inoue et al., 2019; Yamaoka et al., 2021). In contrast to wild type, Mp kan mutants continued to grow plagiotropically under FR-enriched light and exhibited delayed gametangiophore initiation (Fig. S8a–l). The angle formed between the thallus and substrate ($\theta_{TS}$) was compared between Mp kan and wild type under WL and FR. In wild type, $\theta_{TS}$ significantly increased under FR when compared with WL (Fig. 3a). By contrast, Mp kan plants had significantly lower $\theta_{TS}$ under both light conditions than wild type, and $\theta_{TS}$ did not significantly increase under FR (Fig. 3a). In the case of Mp kan-7ge, mean $\theta_{TS}$ values were negative, with the thallus margins growing into the substrate. Thus, Mp kan plants displayed reduced orthotropic growth compared with wild type under both light conditions, and failed to grow orthotropically in response to FR.

We next examined gametangiophore initiation in Mp kan lines. In wild type, the first gametangiophores were produced after 18–20 d in males and 20–22 d in females, with a marked increase in the number of gametangiophores per notch from 22 to 26 d (Fig. 3b). In Mp kan plants, gametangiophore-like tissues were produced, and the appearance of the first reproductive tissues was delayed by 8 d in males and 14 d in females (Fig. 3b). Thus, FR-induced gametangiophore initiation was delayed in the Mp kan mutant background.

Additionally, a number of morphological abnormalities were observed in Mp kan reproductive tissues (Fig. 3c–q). Wild-type gametangiophores develop via modifications of the thallus, giving rise to the two specialised regions of the gametangiophore: the stalk and the gametangium-bearing receptacle. In males, three successive rounds of dichotomous branching produce an 8-lobed receptacle, with apical notches located at lobe tips (Fig. 3c,i,k). Antheridia are produced from dorsal derivatives of the apical cell and from within dorsal cavities of antheridiophore receptacles. The dorsal surface of the antheridiophores receptacle produces both air pores and antheridial pores, with the latter exuding sperm when antheridia are mature (Fig. S9a). In wild-type females, three rounds of dichotomous branching, with occasional extra bifurcations, give rise to archegoniophores possessing 9–11 digitate rays, with apical notches residing between the rays (Fig. 3d,m,p). Archegonia form from dorsal epidermal derivatives of apical cells and are located between the bases of the rays. Archegonia are protected by involucres (Figs 3p, S9d). In both sexes, the receptacles are elevated on stalks that form by inward rolling of the thallus, where the dorsal surface of the thallus envelopes an internalised ventral side producing scales and bundles of rhizoids (Figs 3c,d, S9g,j). No air pores form on the surface of the stalk, and dorsal epidermal cells are columnar in shape (Fig. S9g,j).

In Mp kan lines grown under FR, mature antheridia and archegonia form in male and female lines, respectively, but without characteristic thallus modifications. In both sexes, gametangiophore morphology resembled the wild-type vegetative thallus morphology and lacked the clear distinction between stalk and receptacle tissues. Stalk regions were stunted and not inward-rolled, and columnar-shaped air pores were present (Figs 3e,f,j,n, S9h,i,k,l). Gametangiophore receptacle morphology was also intermediate between wild-type vegetative and gametangiophore morphology (Fig. 3e,f,j,n,o). In Mp kan-5ge males, antheridia formed internally in receptacles that were less lobed than wild-type antheridiophore receptors (Fig. 3e,j,l). Dorsally, most antheridal pores and air pores formed normally (Fig. S9b), but occasionally, antheridium-like structures were observed protruding from large pore-like structures (Fig. S9e). In Mp kan-7ge females, archegoniophores were similarly less modified from the vegetative thallus morphology (Fig. 3f,n,q). Archegonia were produced, but lacked involucres (Figs 3o, S9e,f). Mature Mp kan-5ge and Mp kan-7ge gametangiophores had fertile sperm and egg cells, respectively, as crosses between Mp kan-7ge and Mp kan-5ge produced viable Mp kan-7ge/Mp kan-5ge sporophytes (Fig. S8n,o). Complemented Mp kan-5ge lines of both sexes grown under FR displayed normal gametangiophore development (Fig. 3g,h). Altogether, these results suggest after gametangium initiation, MpKAN regulates the transition from vegetative to sexual tissue types (i.e. gametangiophore formation) under FR.
Fig. 3 Mpkan mutants fail to complete far red (FR)-induced developmental transitions in Marchantia. (a) $\theta_{TS}$ values of 5-d-old gemmalings grown under constant WL (solid bars) and FR (striped bars). Data are mean $\theta_{TS}$ (columns) ± SD. Statistical differences obtained using two-way ANOVA and Tukey’s multiple comparisons tests. Letters denote statistically significant different groups ($P < 0.05$). (b) Ratio of receptacles : apical notches in wild-type and Mpkan FR-induced plants. Data are mean ± SD. (c–h) Comparison between the sexual tissues of wild-type, Mpkan and complemented lines induced under FR-enriched light conditions. Plants were grown under FR from gemmae for 35 d (c–e, g, h) and 45 d (f). (i–q) SEM imaging of dorsal (i, j, m–o) and ventral (k–l, p–q) reproductive tissues (*, other positions where archegonia are evident). Wild-type male (i, k) and female (m, p) and Mpkan male (j, l) and female (n, o, q) thalli were grown for 55 d under FR. Magnified insets depict (o) archegonia and (p) involucres. Panels (i–n) and (p) are composites of SEM images. Bars: (c–h, i–n, p) 1 mm; (o, q) 500 μm.
Effect of inducible MpKAN overexpression on development and FR growth responses

To examine the effects of MpKAN overexpression, DEX-inducible pro\_MpEF1:MpKAN-GR transgenic lines were generated. Mock-treated pro\_MpEF1:MpKAN-GR lines resembled wild-type plants, whereas DEX-treated pro\_MpEF1:MpKAN-GR lines displayed a range of weak-to-strong phenotypic defects (Figs 4a, S10a). DEX-treated pro\_MpEF1:MpKAN-GR germalings were smaller and produced fewer air pores and no gemmae cups, and stronger lines displayed severely reduced apical growth and loss of dorsal differentiation (Figs 4a, S10a–c). pro\_MpEF1: MpKAN-GR plants grown on mock for 20 d and then transferred to DEX-produced gemmae cups with significantly fewer gemmae, with plastochron 4-derived gemmae cups containing almost no gemmae (Fig. S10d–f). DEX-treated pro\_MpEF1:MpKAN-GR lines also displayed abnormal growth tropisms in response to light treatments. Under WL, DEX-treated pro\_MpEF1:MpKAN-GR plants grew more orthotropically than wild type, and comparably with wild-type plants grown under FR (Fig. 4b), indicating that MpKAN overexpression can induce growth tropisms under WL similar to those normally observed under FR. Under FR, DEX-treated pro\_MpEF1:MpKAN-GR lines failed to produce gametangiophores (Fig. S11). Altogether, constitutive overexpression of MpKAN causes a reduction in growth, the number of air pores, gemmae cups and gemmae, and promotes orthotropic growth under both WL and FR.

MpKAN negatively regulates expression of MpTAA and MpDELLA, and has reduced FR-induced changes in gene expression

To investigate downstream targets of MpKAN, we looked at transcriptome changes in whole thallus tissue collected from Mpkan, pro\_MpEF1:MpKAN-GR and wild-type lines. We first identified gene expression upregulated or downregulated in pro\_MpEF1:MpKAN-GR lines compared with wild type after DEX treatment (Table S2). DEG counts in DEX-treated pro\_MpEF1: MpKAN-GR plants revealed a higher number of downregulated than upregulated genes after 3 h of DEX treatment, suggesting that, as expected, MpKAN acts primarily as a transcriptional repressor (Figs S12, S13a,b). The GO analysis of differentially expressed genes revealed that a significantly enriched number of phenylpropanoid/flavonoid biosynthesis genes were downregulated in DEX-treated pro\_MpEF1:MpKAN-GR lines (data from Hernández-García et al., 2021b), both phenotypes that are shared with Mpkan plants. To investigate the link between MpKAN and MpDELLA further, we compared genes upregulated in MpDELLA overexpression lines (data from Hernández-García et al., 2021) with those upregulated in FR-treated Mpkan lines. There was substantial overlap between gene lists (Fig. S13h), with phenylpropanoid and flavonoid biosynthesis-related overrepresentation in the subset of genes upregulated in both MpDELLA-ox and FR-treated Mpkan lines (Fig. S15; Table S4), suggesting that flavonoid biosynthesis genes are shared downstream targets of MpKAN and MpDELLA.

To identify genes downstream of MpKAN activity that may cause reduced FR responses in Mpkan plants, we examined the differences in gene expression between wild-type and Mpkan lines after 5 d in FR light conditions, targeting genes involved in early gametangiophore specification (Fig. 4e–h). FR-upregulated genes in wild-type males and females included MpBONOBO (MpBNB), which encodes a TF necessary for gametangium specification in Marchantia (Yamaoka et al., 2018), as well as the TALE-homeodomain TF-encoding geneMpBELLI, consistent with a previous study (Inoue et al., 2016) (Figs 4e,f, S14a,i). In wild-type females, the regulator of female sexual differentiation, MpFGMYB (Hisanaga et al., 2019), was FR-upregulated (Fig. 4f). Candidate gibberellic acid biosynthesis genes orthologous to those encoding enzymes that catalyse GA12 from GGD, in angiosperms (Figs S14c) (Hedden, 2020), as well as cytokinin biosynthesis genes (MpIPT2 and MpLOG) (Bowman et al., 2017), were also FR-upregulated in wild-type males and females (Figs 4e,f, S14d–g). In Mpkan lines, transcript levels of MpBNB and most candidate GA12 biosynthesis genes were no longer FR-upregulated (Figs 4g,h, S14d–i; Table S3), indicating that FR-induced upregulation of these genes is dependent on MpKAN activity. Gene ontology (GO) analysis indicated that FR-upregulated genes in Mpkan plants were not enriched for gibberellin biosynthesis and metabolism terms, as observed in FR-upregulated genes in wild-type plants (Table S4). In Mpkan lines, however, MpBELLI and the cytokinin biosynthesis genes were still FR-upregulated (Figs 4e–h, S14i), implying independence from MpKAN activity.

MpKAN can rescue the sporophyte polarity defects of Arabidopsis kanadi mutants

In angiosperms KANs, TFs promote a sporophyte abaxial identity (Eshed et al., 2001), whereas MpKAN does not regulate tissue polarity in either generation of Marchantia. To assess whether
Fig. 4  DEX-inducible overexpression of MpKAN and analysis of transcriptional targets of MpKAN in Marchantia. (a) Phenotypes of wild type (left) and proMpEF1: MpKAN-GR (right) transformants treated with mock (top) and 10 µM DEX (bottom). Plants were grown for 9 d. (b) Comparison of angle between thallus and substrate (θTS) of mock and DEX-treated plants. Data are mean (columns) ± SD. Statistical differences were obtained using two-way ANOVA and Tukey’s multiple comparisons tests. Letters denote statistical significant differences (P < 0.05). (c, d) TPM plots of (c) MpTAA and (d) MpDELLA loci from transcriptome profiles of white light (WL)- and far red (FR)-treated wild-type and Mpkan plants. Values associated with brackets are logFC values (adj. P < 0.001). (e-h) Volcano plots showing FR-upregulated and FR-downregulated genes in wild-type (e, f) and Mpkan (g, h) transcriptomes. LogFC values obtained using the edgeR software comparing wild-type FR with wild-type WL (e, males; f, females) and Mpkan FR with Mpkan WL (g, males; h, females). Yellow loci, nonsignificant (adj. P < 0.001); grey loci, −1 < logFC < 1; blue loci, FR-upregulated (logFC > 1) or FR-downregulated (logFC < −1) genes. Transcriptional regulators, peptides/receptors, and phytohormone- and light-related genes (Bowman et al., 2017) were annotated on volcano plots. Genes of interest with logFC > 1 or logFC < −1 are labelled in black. Genes of interest in teal showed same FR response in all sexes and genotypes. Genes of interest in pink showed same FR response in both sexes for one genotype, but not in the other genotype (and are shown in red text in volcano plots of other genotype). Bars, (a) 1 mm.
Table 1  Genes upregulated in Mpkan plants and downregulated in DEX-treated proMpEF1-MpKAN-GR plants in Marchantia.

| v.5.1 ID  | Name                      | Description                                      | Fold change response (log 2FC) |
|----------|---------------------------|--------------------------------------------------|-------------------------------|
|          |                           |                                                  | Mpkan-5th d/WT       | Mpkan-7th d/WT      | pMpEF1/MpKAN-GR/WT |
|          |                           |                                                  | WL  | FR  | WL  | FR  | 3 h DEX | 24 h DEX |
| Mp1g04680| Cytochrome P450            |                                                  | 1.89 | 2.37 | 2.02 | 2.44 | –0.85  | –0.89   |
| Mp1g21930| LOX2S; lipoxygenase        |                                                  | 1.24 | 1.16 | 1.53 | 1.83 | –1.53  | –1.76   |
| Mp1g28830| Unannotated               |                                                  | 4.42 | 6.24 | 3.01 | 5.61 | –2.99  | –3.39   |
| Mp2g02350| Protein of unknown function (DUF3455) |                                              | 2.34 | 3.2  | 1.47 | 3.19 | –1.02  | –1.92   |
| Mp2g03050| Cytokinin receptor         |                                                  | 0.93 | 0.53 | 0.84 | 0.56 | –1     | –0.69   |
| Mp2g03280| 9-cis-beta-carotene 9’,10’ cleaving dioxygenase |                                         | 1.47 | 3.77 | 1.37 | 3.6  | –1.56  | –3      |
| Mp2g13230| Unannotated               |                                                  | 3.02 | 3.47 | 3.07 | 2.46 | –1.62  | –2.43   |
| Mp3g10530| Serine/threonine protein kinase |                                              | 2.82 | 2.2  | 2.64 | 1.38 | –1.43  | –2      |
| Mp3g15790| Peroxidase                |                                                  | 7.27 | 8.01 | 6.17 | 6.31 | –3.99  | –4.49   |
| Mp3g17960| Unannotated               |                                                  | 1.07 | 1.15 | 1.12 | 1.09 | –1.05  | –1.66   |
| Mp3g21740| Peroxidase/oxygenase       |                                                  | 2.2  | 1.88 | 1.16 | 1.1  | –1.1   | –1.62   |
| Mp4g03600| Sulphite exporter Tau/E/SafE |                                              | 2.15 | 2.64 | 2.84 | 3.32 | –1.76  | –1.46   |
| Mp4g12310| Serine/threonine protein kinase |                                          | 0.84 | 0.63 | 0.68 | 0.53 | –0.72  | –0.91   |
| Mp4g15440| Indole-3-acetate O-methyltransferase |                                         | 0.86 | 1.49 | 0.53 | 1.2  | –0.59  | –0.77   |
| Mp4g16270| Unannotated               |                                                  | 1.17 | 1.13 | 1.58 | 0.75 | –0.62  | –0.74   |
| Mp5g00560| Methyltransferase         |                                                  | 3.12 | 2.7  | 3.02 | 1.84 | –1.52  | –2.93   |
| Mp5g06850| O-methyltransferase       |                                                  | 0.67 | 1.3  | 0.71 | 1.47 | –1.1   | –0.69   |
| Mp5g14320| Aminotransferase         |                                                  | 1.94 | 2.48 | 1.55 | 1.82 | –1.15  | –0.64   |
| Mp5g14990| Unannotated               |                                                  | 1.59 | 0.89 | 1.55 | 1.4  | –0.88  | –1.03   |
| Mp5g16240| EamA-like transporter     |                                                  | 3.68 | 3.83 | 3.23 | 2.59 | –2.78  | –2.67   |
| Mp5g20660| Transcription factor, GRAS family |                                      | 0.58 | 1.14 | 0.6  | 1.12 | –1.45  | –0.9    |
| Mp7g01790| 6-methylsalicylate decarboxylase |                                | 1.26 | 1.54 | 1.25 | 1.44 | –0.92  | –0.75   |
| Mp7g08280| Unannotated               |                                                  | 0.53 | 1.24 | 0.63 | 1.41 | –0.6   | –1.45   |
| Mp7g09830| Alpha/beta hydrolase      |                                                  | 1.44 | 1.02 | 1.68 | 1.29 | –0.83  | –0.87   |
| Mp7g11540| 3-phosphoglycerate kinase |                                                  | 1.91 | 1.11 | 2.31 | 1.13 | –0.55  | –1.19   |
| Mp7g14650| 3-methylcrotonyl-CoA carboxylase |                                | 1.08 | 0.76 | 1.55 | 1.16 | –1.05  | –0.65   |
| Mp7g16380| rnc, DROSHA, RNT1; ribonuclease III |                              | 1.21 | 1.32 | 1.72 | 1.82 | –4.36  | –2.49   |
| Mp8g06340| Dirigent-like protein     |                                                  | 1.05 | 0.95 | 1.49 | 1.4  | –0.83  | –1.54   |
| Mp8g11680| Polyketide cyclase/dehydrogenase |                                            | 0.93 | 1.31 | 1.11 | 1.36 | –0.68  | –1.46   |

Gene list of loci with opposite changes in transcript abundance of RNA samples from Mpkan, wild-type and proMpEF1-MpKAN-GR lines; fold changes are measured using EdgeR analysis (adj. P < 0.001). Genes in list meet the following requirements: logFC > 0.5, Mpkan/WT; logFC < –0.5, proMpEF1-MpKAN-GR/WT; and logFC ≥ 0 WT 3 h DEX/WT 0 h mock.

MpKAN can fulfill this role in angiosperms, and whether KAN TFs are conserved at the protein level across land plants, we expressed MpKAN with the Arabidopsis AtKAN1 promoter in kanadi1-2 kanadi2-1 (kan1-2 kan2-1) plants. Remarkably, the phenotype of proAtKAN1: MpKAN kan1-2 kan2-1 plants approaches that of wild type in leaf, inflorescence and flower morphology (Figs 5, S16), indicating that MpKAN can substitute for reduced AtKAN abaxial identity activity in kan1-2 kan2-1 mutants, and demonstrating that MpKAN maintains an AtKAN biochemical function.

Discussion

KANADI TFs are biochemically conserved across land plants

Since the loss of KAN activity in Arabidopsis can be partially complemented by transgenic expression of MpKAN (Figs 5, S16), we conclude that KAN protein structure, and its ability to act with partners, is largely conserved across land plants. However, as discussed below, our results suggest distinct biological roles of KANADI in liverworts and angiosperms, indicating the evolution of either (1) changes in expression domains between lineages or (2) the divergence of downstream transcriptional targets via changes in cis-regulatory binding sites.

MpKAN influences apical growth and dorsal differentiation

In contrast to vascular plants, MpKAN – while expressed in the sporophyte generation – did not have a conspicuous developmental role in the sporophytes of Marchantia (Figs 1, S6). Again in contrast to angiosperms, MpKAN plays a functional role in the gametophyte generation of Marchantia (Fig. 1). MpKAN expression was observed throughout the apical notches in the epidermal and subepidermal files close to the meristematic apical cell (Fig. 1) and is thus expressed where most developmental decisions are occurring. The loss of MpKAN activity resulted in more rapid bifurcations during the first plastochron, as well as reduced tissue differentiation (Figs 2, S6). Constitutive expression of MpKAN caused strong suppression of apical growth and the loss of gemmae production in developing gemmae cups, the latter
process involving meristematic gemma initials (Figs 4, S10). Taken together, these results suggest that MpKAN is either (1) a negative regulator of meristem activity in the apical notch; (2) a positive regulator of differentiation of subepidermal and epidermal cells as they are displaced from the apical cell; or (3) a combination of both roles. Given that two key meristem-associated genes – MpTAA and MpCLE1 (Eklund et al., 2015; Hirakawa et al., 2019) – were downregulated in DEX-treated proMpEFI: MpKAN-GR plants (Tables 1, S2), one might presume that MpKAN has at least the first of these roles.

In angiosperm sporophytes, KAN members regulate radial patterning by promoting abaxial and peripheral tissue types in the lateral organs and shoot (Eshed et al., 2001, 2004; Kerstetter et al., 2001; Emery et al., 2003; Izhaki & Bowman, 2007). In the gametophyte generation of Marchantia, MpKAN is not expressed in a polarised manner, and Mpkan plants do not display defects in patterning along spatial axes (Figs 1–2, S6). Additionally, Mpkan-5β/Mpkan-7β homozygous sporophytes did not display tissue polarity defects (Fig S6). Thus, in contrast to KAN orthologs in angiosperms, MpKAN is not required for coordinating tissue polarity in either generation in Marchantia, suggesting that a role in patterning along spatial axes may have evolved de novo within vascular plant sporophytes, or was lost in bryophytes – a scenario that is equally parsimonious if bryophytes are monophyletic (Puttick et al., 2018).

The function of KAN members in Arabidopsis embryo development goes beyond tissue polarity, as kan triple mutants show ectopic organ formation, where KAN TFs suppress organogenesis via the repression of PIN1-FORMED (PIN1) auxin transporters (Izhaki & Bowman, 2007). This bears resemblance to the role of
MpKAN in the Marchantia gametophyte generation, with MpKAN-mediated thallus differentiation potentially connected with its capacity to downregulate Mp TAA and cause auxin depletion in Marchantia (Figs 4, S7). Indeed, the loss of MpKAN function in Marchantia phenocopies auxin-treated wild-type plants possibly due to the regulation of Mp TAA. Further functional analyses in bryophytes and seedless tracheophytes could corroborate the deep conservation of this function across land plants.

MpKAN modulates developmental FR responses, including the transition to sexual reproduction

MpKAN functions in multiple aspects of development in response to FR in Marchantia. First, Mpkan thalli displayed reduced orthotropic growth compared with wild type under both WL and FR (Fig. 3). Conversely, MpKAN overexpression caused orthotropic growth under both WL and FR (Fig. 4), suggesting MpKAN may be sufficient to initiate FR-induced orthotropic growth. Similarly, Mp phy mutants grow orthotropically under both WL and FR, suggesting that MpPHY inhibits orthotropic growth (Inoue et al., 2019). Thus, the plagiotropic growth form of Mpkan mutants under both light treatments may reflect reduced phytochrome-mediated growth tropisms. While not homologous structures, in Arabidopsis leaves phytochrome (PHYB) and KAN activities also affect leaf flatness, that is plagiotropic growth, in an opposite manner (Kozuka et al., 2013; Johansson & Hughes, 2014). Second, Mpkan mutants exhibited a delayed transition to sexual reproduction following FR treatment. In wild type, FR-upregulated genes included MpBNB, a master regulator of gametangium initiation in Marchantia that is required for subsequent gametangiophore formation (Yamaoka et al., 2018). MpBNB transcripts were not FR-upregulated in Mp kan plants (Fig. 4), suggesting that the loss of MpKAN activity causes a delay in the transition to sexual reproduction via delayed activation of MpBNB. Third, Mpkan mutants produced vegetative-like gametangium-bearing structures, with the Mpkan reproductive thallus morphologically intermediate between wild-type vegetative thallus tissue and wild-type gametangiophore tissues (Fig. 3). Given that gametangiophores form only after MpBNB-mediated gametangium initiation (Yamaoka et al., 2018, 2021), this would suggest that MpBNB activity is not entirely lost in Mp kan plants and that after gametangium specification, MpKAN is additionally required for gametangiophore formation under FR. One might predict that MpKAN overactivity would cause early or ectopic formation of gametangiophores; however, MpKAN overexpression caused complete suppression of gametangiophore formation (Fig. S11), likely due to the previously mentioned role as a negative regulator of meristem activity.

MpBNB physically interacts with PHYTOCHROME-INTERACTING FACTOR (MpPIF), with both transcription factors acting in a pathway-regulating gametangium and gametangiophore formation under FR (Inoue et al., 2019; Hernández-García et al., 2021b). FR-treated Mpkan plants expressed elevated levels of MpDELLA, and MpDELLA and MpKAN similarly influence gene expression involved in flavonoid biosynthesis (Figs 4, S15). Thus, we postulate that MpKAN and MpDELLA may act in a genetic pathway wherein MpKAN negatively regulates MpDELLA, which in turn inhibits the activity of MpPIF, whose activity promotes both the transition to sexual reproduction and flavonoid biosynthesis (Fig. 6). However, MpPIF and MpKAN possess distinct roles in directing

![Fig. 6](https://example.com/figure6.png)

Fig. 6 Theoretical pathways for the regulation of far-red (FR) responses by KAN transcription factors (TFs) in land plants. Components with yellow background are proposed to be conserved in land plants, ones with white background are not conserved, and those with green background have insufficient information to determine whether pathway position is conserved in land plants. Arrows and blunt-ended arrows indicate positive and negative regulations, respectively. (a) In Marchantia, MpKAN regulates MpDELLA expression, which in turn negatively regulates MpPIF activity. Under high FR, MpPHY enters the nucleus and positively regulates MpPIF, which promotes the transition to sexual reproduction via gametangium initiation. (b) In Arabidopsis, phytochromeB (phyB) is nuclear localised under high R : FR, where it negatively regulates PIF activity via (1) protein–protein interactions resulting in degradation via the ubiquitin–proteasome system; and (2) reduced GA levels, which frees the DELLA transcription factor AtRGA to repress AtPIF4 and AtPIF5 activity. Under low R : FR (shade), phyB is located in the cytoplasm, resulting in increased PIF activity. PIF and KAN TFs act antagonistically to regulate expression of AtTAA1 expression, which in turn promotes hypocotyl elongation. Theoretical pathways are formulated based on results of this paper and previous work (Casal, 2013; Merelo et al., 2017; Inoue et al., 2019; Hernández-García et al., 2021b). Question mark indicates partially supported pathway step where only AtKAN1 negatively regulates AtRGA (Merelo et al., 2013).
Loss of KANADI activity disrupts FR-induced expression of GA12 biosynthesis genes

In wild-type Marchantia, FR-upregulated genes included orthologs of cis-Zeatin and GA12 biosynthesis genes in Arabidopsis, making the activity of both molecules candidates for mediating FR responses. In M. polymorpha, GA12 biosynthesis genes were no longer upregulated in response to FR (Figs 4, S14). AtKAN4 also suppresses GA biosynthesis, including GA12 production (Gomez et al., 2016). Bryophytes are unlikely to produce canonical bioactive GAs found in angiosperms and do not possess GID1 receptor orthologs, which in vascular plants are required for interactions between GA and DELLA proteins (Hernández-Garcia et al., 2021a). In one plausible scenario, in the ancestral land plant, KAN TFs regulated DELLA activity and GA production independently, and in the vascular plant ancestor, the DELLA pathway became GA-dependent via acquisition of the GID1 receptor. Alternatively, in the ancestral land plant KAN TFs regulated a GID1-DELLA-GA pathway, and in the ancestral bryophyte GID1 orthologs were lost, decoupling DELLA from GA regulation.

KANADI TFs – conserved regulators of developmental FR responses

In flowering plant sporophytes, KAN TFs regulate both tissue polarity and avoidance responses via common gene targets (Merelo et al., 2017). Here, we found that MpKAN does not regulate tissue polarity in either generation of the Marchantia life cycle and instead functions in at least two contexts during the gametophyte generation. In the context of vegetative development, MpKAN is active in the derivatives of the apical cell, where it regulates thallus apical growth and tissue differentiation. In the context of FR-enriched light treatment, MpKAN modulates multiple FR responses including MpBNB-mediated gametangiophore initiation and positively regulates gametangiophore formation after the gametangium initiation have been specified. While acting in alternating generations, KAN TFs may have conserved roles as modulators of FR responses in both angiosperms and liverworts, a function presumably present in the ancestral land plant.

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Author contributions

LNB, EF-S and JLB designed the research; LNB, JPA, TD and EF-S performed research and analysed data; LNB and JLB wrote the paper with input from all authors.

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Data availability

The data supporting the findings of this study are available upon request from the corresponding author. All RNA-seq data used in this study are openly available and accessible as FASTQ files in SRA (NCBI) under the following Biosample accession nos.: SAMN21246197, SAMN21246198, SAMN21246199, SAMN21246200 and SAMN21246201, Bio-project ID: PRJNA761216.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Promoter, gene and protein sequences of MpKAN.

Fig. S2 Sequence alignments of wild-type and Mpakan loss-of-function alleles.

Fig. S3 Principle component analysis of RNA-seq samples.

Fig. S4 Expression of MpKAN in vegetative and reproductive tissue types.

Fig. S5 Genotyping alleles of Mpakan loss-of-function and complemented lines.

Fig. S6 Morphology of wild-type and Mpakan plants grown under white light.

Fig. S7 Loss of MpKAN activity may cause altered auxin distributions.

Fig. S8 Loss of MpKAN activity causes altered responses to FR light.

Fig. S9 Scanning electron micrography of wild-type and Mpakan reproductive tissues.

Fig. S10 Effect of constitutive over-expression of MpKAN.

Fig. S11 Effect of constitutive MpKAN overexpression on sexual reproduction.

Fig. S12 RNA-seq analysis: Differentially expressed gene counts.

Fig. S13 Volcano plots of gene loci.

Fig. S14 TPM plots of differentially expressed gene loci of interes.

Fig. S15 Heatmap of expression changes in flavonoid biosynthesis genes.

Fig. S16 MpKAN can rescue the flower and silique defects of Arabidopsis kanad1 mutants.

Methods S1 Supplementary Materials and Methods.

Table S1 Primers used in this study.
Table S2 RNA-seq data.

Table S3 RNA-seq data of FR-treated wild-type and Mpkan plants.

Table S4 GO enrichment analysis.

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