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Evolutionary-conserved RLF, a cytochrome *b*₅-like heme-binding protein, regulates organ development in *Marchantia polymorpha*

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Summary

- In *Arabidopsis thaliana*, REDUCED LATERAL ROOT FORMATION (RLF), a cytochrome *b*₅-like heme-binding domain (Cytb5-HBD) protein, is necessary for proper lateral root (LR) formation. Whereas the other Cytb5-HBD proteins in *A. thaliana* regulate different metabolic reactions, RLF is unique as it specifically regulates organ development. However, it remains unknown whether heme binding to RLF is necessary for its function and whether RLF orthologs in different plant species also regulate organ development.
- We demonstrate that RLF binds to heme *in vitro* and that two histidine residues, which are conserved among Cytb5-HBD, are crucial for both heme binding and its biological function in *A. thaliana*. In addition, we show that MpRLF, a RLF ortholog in the bryophyte *Marchantia polymorpha*, also binds to heme *in vitro* and that MpRLF rescues the LR formation phenotype of the *A. thaliana* *rlf* mutant.
- *Mprl^{fge}*, the loss-of-function mutation in MpRLF, resulted in delayed thallus growth and inhibited both gemma cup and reproductive organ formation.
- Our findings indicate that MpRLF is essential for proper vegetative and reproductive development in *M. polymorpha*. This suggests that RLF-dependent redox reaction systems are conserved across diverse plant species and were independently co-opted for organ development in bryophyte and seed plant evolution.

Introduction

Heme is a porphyrin complex with a centrally coordinated iron atom. In plants, heme shares a metabolic pathway with Chl biosynthesis up to the production of protoporphyrin IX, after which ferrochelatase coordinates Fe²⁺ to protoporphyrin IX (Roper & Smith, 1997; Hederstedt, 2012). In the Protein Data Bank (<https://www.rcsb.org>), which contains 222 415 proteins, 4272 protein chains have been identified as heme-binding proteins (HBPs) from entries containing heme types *b* and *c* (Li *et al.*, 2011). The binding of these HBPs to heme is maintained by the coordination of specific amino acids, such as histidine, within the apoprotein to the iron atoms of heme. In general, in various organisms, HBPs are essential for diverse biological processes such as steroid biosynthesis, aerobic respiration, and programmed cell death because of their role in electron transfer, substrate oxidation, and metal ion storage (Reedy & Gibney, 2004; Layer *et al.*, 2010). In plants, HBPs, such as cytochrome *c*, which is involved in electron transfer during

photosynthesis, and SOUL4, which is implicated in lipid metabolism within chloroplast fat droplets (plastoglobule), have demonstrated diverse physiological effects (Kerfeld & Krogmann, 1998; Shanmugabalaji *et al.*, 2020). Recently, proteomic analyses in *Arabidopsis thaliana* have identified a variety of HBPs, such as basic/helix–loop–helix type nuclear transcription factors and intracellular signaling factors involved in GTPase activation (Shimizu *et al.*, 2020). These studies have revealed that HBPs function as intracellular signals regulating diverse physiological functions. However, it remains unclear how they are involved in plant organ development.

One group of HBPs in plants is a family of proteins with a cytochrome *b*₅-like heme-binding domain (Cytb5-HBD). In plants, the metabolic pathways mediated by most Cytb5-HBD proteins are diverse, including fatty acid desaturation, lignin biosynthesis, and nitrate reduction (Nagano *et al.*, 2012; Gou *et al.*, 2019). The genome of *A. thaliana* has 15 proteins with a Cytb5-HBD, including five members of the cytochrome *b*₅ family (At1g26340 (CB5A), At2g32720 (CB5B), At2g46650

(CB5C), At5g48810 (CB5D), and At5g53560 (CB5E)) (Maggio *et al.*, 2007), one cytochrome *b₅*-like protein (At1g60660 (CB5LP)), four membrane-associated progesterone binding proteins (At2g24940 (MAPR2), At3g48890 (MAPR3), At4g14965 (MAPR4), and At5g52240 (MAPR5)) (Yang *et al.*, 2005), two Δ -8 sphingolipid desaturases (At3g61580 (SLD1) and At2g46210 (SLD2)) (Sperling *et al.*, 1998), two nitrate reductases (At1g77760 (NR1) and At1g37130 (NR2)) (Cheng *et al.*, 1988; Wilkinson & Crawford, 1991) and At5g09680/REDUCED LATERAL ROOT FORMATION (RLF) (Ikeyama *et al.*, 2010). In *A. thaliana*, cytochrome *b₅* isoforms, SLD1, SLD2, NIA1, NIA2, and proteins of the MAPR family generally do not play a significant role in organ development. CB5D is involved in lignin biosynthesis, and the *cb5d* mutant shows a reduction in S-lignin content. However, no abnormalities in organ development have been observed in *cb5d* mutants (Gou *et al.*, 2019). SLD1 and SLD2 are involved in Δ -8 sphingolipid desaturation. Their loss-of-function mutants show significantly reduced root growth under sodium dodecyl sulfate (SDS) stress, but no growth defects are observed under normal conditions (Chen *et al.*, 2012). Similarly, NIA1 and NIA2 are involved in the regulation of nitrate metabolism and nitric oxide (NO) production; however, the null-allelic mutant *nial1-3 nia2-1* is lethal, and no studies have reported effects on organ development. Additionally, single mutants of either *NIA1* or *NIA2* do not exhibit significant effects on plant growth or development (Wilkinson & Crawford, 1993; Tang *et al.*, 2022). Membrane-associated progesterone binding proteins, particularly MAPR5, contribute to lipid and steroid metabolism and regulate autophagy under starvation conditions, potentially influencing energy homeostasis. However, no severe developmental abnormalities have been reported in MAPR mutants or overexpression lines (Kimura *et al.*, 2012; Ryu *et al.*, 2017; Wu *et al.*, 2021).

Whereas most of the Cytb5-HBD proteins in *A. thaliana* regulate a variety of metabolic reactions, At5g09680/RLF is a unique Cytb5-HBD protein that regulates organ development, including lateral root (LR) formation (Ikeyama *et al.*, 2010). In general, LR formation is essential for root system architecture in most vascular plants and is regulated by auxin. In *A. thaliana*, LR formation is regulated by several auxin signaling modules including the SOLITARY-ROOT/IAA14, an auxin/indole-3-acetic acid (Aux/IAA) protein – AUXIN RESPONSE FACTOR (ARF) 7 and ARF19 module (Fukaki *et al.*, 2005; Okushima *et al.*, 2007). The *rlf* loss-of-function mutants of *RLF* show a marked reduction in the number of LRs while their ARF7/19-mediated auxin signaling is not affected, suggesting that *RLF* regulates LR formation independently of auxin response (Ikeyama *et al.*, 2010). In addition, the *rlf* mutations cause aberrant organ development, including reduced primary root growth and decreased leaf size (Ikeyama *et al.*, 2010). While the study of *RLF* in *A. thaliana* sheds light on the significance of Cytb5-HBD proteins for plant growth and development, a crucial gap remains in our understanding of how Cytb5-HBD proteins orchestrate growth and development of plant organs. First, it is not known whether *RLF* binds to heme and works as HBP

in *planta*. Second, there is limited understanding of whether Cytb5-HBD proteins share a common role in regulating organ development in land plants. To address these issues, we extend our investigation of *RLF* to the model bryophyte species, *Marchantia polymorpha* (Ishizaki *et al.*, 2016; Fernandez-Pozo *et al.*, 2022) in addition to *A. thaliana*. *M. polymorpha* forms distinct organs specialized for vegetative or reproductive functions, such as the thallus, which serves as the main photosynthetic structure, gemma cups that produce gemmae, and reproductive organs like antheridiophores and archegoniophores. Its genetic tractability and evolutionary position as a nonvascular plant make it an ideal model for exploring these questions.

In this study, we demonstrate that the heme-binding ability of *RLF* is necessary for its biological function. In addition, we unveil the role of *MpRLF*, an ortholog of *RLF* in *M. polymorpha*, in orchestrating proper vegetative and reproductive development. Our findings highlight the crucial role of the *MpRLF* gene in plant organ development. Notably, the similarities between *MpRLF* and *A. thaliana* *RLF* suggest the existence of a conserved mechanism, governed by *RLF* that intricately regulates organ development across diverse plant lineages.

Materials and Methods

Plant materials and growth conditions

Marchantia polymorpha L. accessions Takaragake-1 (Tak-1; male) and Takaragake-2 (Tak-2; female) were used as the wild-type (WT) in this study (Ishizaki *et al.*, 2008). *Marchantia polymorpha* plants were generally cultured on a 1/2-strength Gamborg's B5 medium containing 1% (w/v) agar under continuous light (50–60 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at 20°C. The thalli were imaged using a dissecting microscope and measured using the Fiji imaging software (Schindelin *et al.*, 2012). Observations of gemma cups and reproductive organs were performed on the cultured thalli of *M. polymorpha* using a digital microscope (VHX-5000; Keyence, Osaka, Japan). To observe rhizoid growth, plants were cultivated in an inverted orientation, with the medium placed on the upper side. Observations of rhizoids were conducted using an M205 FA stereomicroscope (Leica Microsystems, Wetzlar, Germany) equipped with a CCD camera (DFC7000 T; Leica Microsystems). For scanning electron microscopy, thalli were frozen in liquid nitrogen and observed with a digital microscope (VHX-D510; Keyence).

Arabidopsis thaliana L. accession Columbia (Col-0) was used as WT in this study. The *rlf* mutant and *RLFpro:RLF-Green Fluorescent Protein (GFP)* transgenic line have been described previously (Ikeyama *et al.*, 2010). Seeds were germinated under sterile conditions on a Murashige & Skoog (MS) medium containing 1% (w/v) sucrose solidified with 0.5% (w/v) gellan gum, as described previously (Goh *et al.*, 2012). *Arabidopsis thaliana* plants were grown at 23°C under continuous light (40–50 $\mu\text{mol m}^{-2} \text{s}^{-1}$). The number of LRs and root length were determined using a dissecting microscope and the Fiji imaging software (Schindelin *et al.*, 2012).

All data are the mean values for each plant considered. Experiments were performed three times, and similar values were obtained in each experiment. Statistical significance of the data was calculated using the Tukey–Kramer test. Statistical analysis of thallus area growth was performed using Dunnett's test, with WT as the control, and adjusted *P*-values were calculated using the single-step method. Significance levels were set as follows: ***, *P* < 0.001; **, *P* < 0.01; *, *P* < 0.05; and †, *P* < 0.1.

Constructions of vectors and generation of transgenic *A. thaliana*

Primers used for plasmid construction are listed in Supporting Information Table S1. To generate *RLFpro:RLF-GFP*, *RLFpro:RLF(H161G)-GFP*, *RLFpro:RLF(H184G)-GFP*, and *RLFpro:RLF(H161G/H184G)-GFP* constructs, the promoter region of *RLF* (*RLFpro*) (804 bp) was amplified from *A. thaliana* WT genomic DNA using IF-pGWB501RLFpro-F and IF-pGWB501RLFpro-R primers and cloned into pGWB501 using the In-Fusion technology (pGWB501 *RLFpro*; Takara Bio Inc., Shiga, Japan). Using the previously synthesized coding sequence (CDS) of full-length *RLF* (*RLFcds*) (Ikeyama *et al.*, 2010) as a template, the *CACC-RLFcds-XhoI* fragment was amplified using *CACC-RLF-F* and *RLF-XhoI-R* primers and cloned into the pENTR/D-TOPO cloning vector (Thermo Fisher Scientific, Rockville, MD, USA) to produce the pENTR-*RLF-XhoI* vector. The *XhoI*-GFP-SalI fragment was obtained from pBluescript II SK(+) GFP by restriction enzyme treatment and ligated with restriction enzyme-cleaved pENTR-*RLF-XhoI* to generate pENTR-*RLF-GFP*. pENTR-*RLF(H161)-GFP* and pENTR-*RLF(H184)-GFP* fragments were created by using PrimeSTAR Max DNA Polymerase (Takara Bio Inc.) and the primers H161G-F/H161G-R and H184G-F/H184G-R, respectively. In addition, the pENTR-*RLF(H161G/H184G)-GFP* fragment was created in the same way using H184G-F/H184G-R primers with pENTR-*RLF(H161)-GFP* as the template. These entry vectors and pGWB501-*RLFpro* were treated with the Gateway LR clonase II enzyme mix (Thermo Fisher Scientific) to generate *RLFpro:RLF-GFP*, *RLFpro:RLF(H161G)-GFP*, *RLFpro:RLF(H184G)-GFP*, and *RLFpro:RLF(H161G/H184G)-GFP* constructs. To generate the *RLFpro:MpRLF*, pGWB501-*RLFpro* and pENTR-*MpRLFcds* vectors (to be described later) were reacted with Gateway LR clonase II enzyme mix (Thermo Fisher Scientific). These binary vectors were transformed into the *rlf-1* mutant using the floral dip method (Clough & Bent, 1998) with *Agrobacterium tumefaciens* (strain C58MP90).

Constructions of vectors and generation of transgenic *M. polymorpha*

Primers used for plasmid construction are listed in Table S1. To generate the *Mp4g04390/MpRLFpro:GUS* construct, the promoter region of *MpRLF* (*MpRLFpro*) was amplified by PCR from Tak-1 genomic DNA as a 4411-bp sequence containing the start codon in the first exon using the primers *MpRLFpro-CACC-F* and *MpRLFpro-R*. The amplified fragment was cloned into the

pENTR/D-TOPO cloning vector (Thermo Fisher Scientific) to generate the pENTR-*MpRLFpro* vector. The *MpRLFpro* region of the pENTR-*MpRLFpro* vector was introduced into the Gateway binary vector pMpGWB104 (Ishizaki *et al.*, 2015) using the Gateway LR clonase II Enzyme mix (Thermo Fisher Scientific) to generate the *MpRLFpro:GUS* binary construct. The *MpRLFpro:GUS* vector was introduced into regenerating thalli of Tak-1 using *A. tumefaciens* GV2260 as previously described (Kubota *et al.*, 2013). Transformants were selected using 100 µg ml⁻¹ hygromycin B and 100 µg ml⁻¹ cefotaxime.

Loss-of-function mutants of *MpRLF* (*Mprlf^{sc}*) were generated using the CRISPR/Cas9 system as described previously (Sugano *et al.*, 2018; Sugano & Nishihama, 2018). We selected the target sequence at the junction of the second intron and the third exon of *MpRLF*. Synthetic oligonucleotides for the target sites shown in Table S1 were annealed, inserted into BsaI-digested pMpGE_En03 using T4 DNA Ligase (Takara Bio Inc.), and then introduced into the destination vector pMpGE010 or pMpGE011 using the Gateway LR clonase II Enzyme mix (Sugano *et al.*, 2018; Sugano & Nishihama, 2018). The vectors were introduced into regenerating thalli of Tak-1 or gemmae of Tak-2 using *Agrobacterium tumefaciens* GV2260 (Kubota *et al.*, 2013; Tsuboyama *et al.*, 2018), and transformants were selected using 100 µg ml⁻¹ hygromycin or 0.5 µM chlorsulfuron.

For the complementation of *Mprlf^{sc}*, the cds of the full-length *MpRLF* was amplified by reverse transcriptase polymerase chain reaction using KOD-Plus-Neo (TOYOBO, Osaka, Japan) with the primers *MpRLFcds-CACC-F* and *MpRLFcds (+stop)-R*. The *MpRLF* coding sequence fragment was cloned into the pENTR/D-TOPO cloning vector (Thermo Fisher Scientific) to produce pENTR-*MpRLFcds*. To prevent CRISPR-Cas9 targeting *MpRLFcds*, a synonymous sequence substitution was introduced between nucleotide positions 472 and 492 (amino acids from G158 to R164) in *MpRLFcds* as follows. pENTR-*MpRLFcds* was used as a template, and the entire plasmid was amplified using PrimeSTAR Max DNA Polymerase (Takara Bio Inc.) and the primers IF-*MpRLF(com)-F* and IF-*MpRLF(com)-R* to produce pENTR-*MpRLFcds_resistant*, a CRISPR-Cas9-resistant *MpRLFcds*. The *MpRLF* promoter region (*MpRLFpro*), including 4408-bp upstream of the initiation codon, was amplified from Tak-1 genomic DNA by PCR using PrimeSTAR Max (Takara Bio Inc.) with the primers IF-*MpRLFpro-F* and IF-*MpRLFpro-R*. The PCR-amplified product was cloned into the HindIII sites of pMpGWB301 (Ishizaki *et al.*, 2015) using the In-Fusion HD cloning kit (Clontech, Mountain View, CA, USA). The CRISPR-Cas9-resistant *MpRLF* coding sequence in pENTR-*MpRLFcds_resistant* was introduced into the binary vector using the Gateway LR clonase II Enzyme mix (Thermo Fisher Scientific) to generate the *MpRLFpro:MpRLFcds_resistant* construct. The *MpRLFpro:MpRLFcds_resistant* vector was introduced into the gemmae of *Mprlf^{sc}* mutants using *A. tumefaciens* GV2260. Detailed methods are described in a previous study (Tsuboyama *et al.*, 2018).

To generate *MpE2Fpro:XVE>MpRLFcds*, the *MpRLF* coding sequence was amplified by PCR using PrimeSTAR Max

(Takara Bio Inc.) with the primer set MpRLFcds-CACC-F and MpRLFcds(–stop)-R and cloned into the pENTR/D-TOPO cloning vector. Lateral root reactions were performed on the C-terminal side of the GFP fusion destination vectors based on the Gateway binary vector pMpGWB168 (Ishida *et al.*, 2022) and entry vectors. The MpE2Fpro:XVE > MpRLFcds-GFP vector was introduced into regenerating thalli of Tak-1 as described previously (Kubota *et al.*, 2013). Transformants were selected with 100 µg ml^{−1} hygromycin B and 100 µg ml^{−1} cefotaxime. To evaluate the effects of conditional overexpression of MpRLF, gemmae of MpE2Fpro:XVE > MpRLF-GFP plants were cultured for 7 d on 1/2-strength Gamborg's B5 medium plates, followed by 7 d on 1/2-strength Gamborg's B5 medium plates containing 10 µM β-estradiol (EST; FUJIFILM Wako Pure Chemical Corp., Osaka, Japan).

To generate the MpRLFpro:RLFcds construct, RLF's full-length coding sequence (RLFcds) was amplified using PrimeSTAR Max DNA Polymerase (Takara Bio Inc.) with the primer set CACC-RLF-F and RLF-stop-R. The previously synthesized RLFcfs was used as a template for amplification (Ikeyama *et al.*, 2010). The amplified RLFcfs fragment was introduced into the pENTR/D-TOPO (Thermo Fisher Scientific) vector via a TOPO reaction to create RLFcfs/pENTR. Using RLFcfs/pENTR and the binary vector pMpGWB301 containing the MpRLF promoter region (MpRLFpro), the MpRLFpro:RLFcfs construct was generated with the Gateway LR Clonase II Enzyme Mix (Thermo Fisher Scientific). The MpRLFpro:RLFcfs vector was transformed into the gemmae of Mp rlf^{fe} mutants using *A. tumefaciens* GV2260. Detailed methods are described in a previous study (Tsuboyama *et al.*, 2018).

GUS staining

For histological β-Glucuronidase (GUS) activity assays, 14-d-old thalli and reproductive organs (induced by 3 wk of white light followed by 7 wk of far-red light and white light) of MpRLFpro:GUS/Tak-1 were de-aerated twice for 5 min, incubated in GUS staining solution (0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 1 mM X-Gluc) at 37°C for 4 h. The samples were then cleared with 70% (v/v) ethanol. Gemmae were incubated in the same GUS staining solution for 24 h under the same conditions. β-Glucuronidase-stained thallus samples were embedded in 6% (w/v) agar. Sections of 100–130 µm samples were cut using a MicroSlicer (DOSAKA DTK-1000; Dosaka EM Co., Ltd, Kyoto, Japan). β-Glucuronidase-stained archegoniophores were dehydrated and embedded in Technovit 7100 resin (Heraeus-Kulwer, Wehrheim, Germany) according to the manufacturer's protocol. Thin sections (5 µm) were cut using a microtome (HM325; MICROM International Inc., Walldorf, Germany), dried, and counterstained for cell walls with 0.05% ruthenium red (Nacalai Tesque Inc., Kyoto, Japan). The sections were imaged by differential interference contrast microscopy (Leica DM6000 microscope; Leica Microsystems) and assembled by e-Tilling (Mitani Shoji Co., Ltd., Tokyo, Japan).

Confocal laser scanning microscopy

The gemmae expressing EST-inducible MpRLF-GFP were counterstained with 10 µg ml^{−1} of propidium iodide (PI) for 5 min and observed by confocal laser scanning microscopy using an FV1000 confocal microscope (Olympus/Evident, Tokyo, Japan). The 473-nm line and the 559-nm line of an LD laser were used to excite GFP and PI, respectively. Images were acquired using the UPlanSAPO 40x/0.95 objective (Olympus/Evident). Images were processed using the Fiji/IMAGEJ program (Schindelin *et al.*, 2012).

Multiple sequence alignments and phylogenetic analysis

The amino acid sequence of *A. thaliana* RLF (At5g09680) was used as a template to identify RLF orthologs in other plant species using NCBI's PSI-BLAST (Position-Specific Iterative BLAST; <https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=psiblast>; Altschul *et al.*, 1997), BLASTP from PHYTOZOME (<https://phytozome-next.jgi.doe.gov/>; Goodstein *et al.*, 2012), and transcriptomic data from the 1000 Plant Transcriptomes Project (<https://db.cngb.org/onekp/>; Leebens-Mack *et al.*, 2019). The RLF orthologs identified in each plant and algae species are listed in Table 1, and their sequences were subjected to multiple sequence alignment using MAFFT. Additionally, the amino acid sequence of *A. thaliana* CB5-D (At5g48810) was used as a template to identify similar sequences of CB5 proteins (CB5A, B, C, D, E, and LP) in other plant species. The RLF and CB5 ortholog sequences from each plant were selected based on an E-value threshold of 1e-15. Multiple sequence alignment of the obtained amino acid sequences was performed using CLUSTALW (Thompson *et al.*, 1994; Waterhouse *et al.*, 2009; Katoh & Standley, 2013). Subsequently, an unrooted phylogenetic tree was built based on the CytB5 HBD amino acid sequences of RLF and CB5 proteins (CB5A, B, C, D, E, and LP) from *A. thaliana* (At5g09680), *Glycine max* (Wm82.a6 Glyma.12G009100 and Glyma.09G227600), *Oryza sativa* (Os07g0232200), *Amborella trichopoda* (AmTr_v1.0_scaffold00029.68), *Thuja plicata* (Thupl.29380466s0005), *Selaginella moellendorffii* (127 066 and 138 045), *Marchantia polymorpha* (Mp3g04390), *Physcomitrium patens* (Pp6c20_12240V6), *Phaeoceros carolinianus* (RXRQ_scaffold_2017981), *Chlamydomonas reinhardtii* (Cre13.g574800), *Volvox carteri* (Voccar.0001s0218), *Chara braunii* (CBR_g29320), and *Porphyra umbilicalis* (Pum0348s0012). Each clade in the phylogenetic tree is labeled according to its classification (e.g. RLF clade, CB5A/C clade). The tree was constructed using the maximum likelihood method (1000 bootstrap tests), the WAG (Whelan and Goldman) model (Whelan & Goldman, 2001), and the MEGA11 software (Tamura *et al.*, 2021). Bootstrap values (1000 tests) are shown as color-coded nodes, indicating their respective confidence levels. The molecular phylogenetic tree was constructed using tvBOT (Xie *et al.*, 2023). The evolutionary distances are displayed below the branches.

The amino acid sequence similarity between RLF and MpRLF was evaluated using EMBOSS Matcher for the full-length

Table 1 Number of REDUCED LATERAL ROOT FORMATION (RLF) orthologs identified in various plant and algae species.

Species	Version	Group	RLF ortholog
<i>Arabidopsis thaliana</i>	TAIR10 (Arabidopsis Information Resource)	Angiosperms	1
<i>Glycine max</i>	Wm82.a6.v1 (PHYTOZOME)	Angiosperms	2
<i>Oryza sativa</i>	v.7.0 (PHYTOZOME)	Angiosperms	1
<i>Amborella trichopoda</i>	v.1.0 (PHYTOZOME)	Angiosperms	1
<i>Thuja plicata</i>	v.3.1 (PHYTOZOME)	Gymnosperms	1
<i>Selaginella moellendorffii</i>	v.1.0 (PHYTOZOME)	Lycophytes	2
<i>Marchantia polymorpha</i>	v.7.1 (MarpolBase)	Liverworts (Bryophytes)	1
<i>Physcomitrella patens</i>	v.6.1 (PHYTOZOME)	Mosses (Bryophytes)	1
<i>Phaeoceros carolinianus</i>	RXRQ (1000KP)	Hornworts (Bryophytes)	1
<i>Chlamydomonas reinhardtii</i>	CC-4532 v6.1 (PHYTOZOME)	Green Algae	1
<i>Volvox carteri</i>	v.2.1 (PHYTOZOME)	Green Algae	1
<i>Chara braunii</i>	Cbr_1.0 (NCBI)	Charophytes	1
<i>Porphyra umbilicalis</i>	v.1.5 (PHYTOZOME)	Red Algae	1

Each row includes the species name, version, group classification, and the number of corresponding RLF orthologs.

sequence, N-terminal region, and CytB5 HBD (https://www.ebi.ac.uk/jdispatcher/psa/emboss_matcher; Madeira *et al.*, 2024).

Protein purification and measurement of absorbance spectra

Purification of recombinant proteins was performed according to the protocol of the pMAL Protein Fusion and Purification System (New England Biolabs, Ipswich, MA, USA). The expression clone maltose-binding protein (MBP)-RLF was obtained by an LR reaction of the pMal-c5X Vector, which contains the *attR1* sequence, *ccdB* gene, and *attR2* sequence at the multicloning site, with *RLFcds*/pENTR. To generate MBP-mutant RLF, we generated the constructs *RLFcds*(H161A)/pENTR, *RLFcd*(H184A)/pENTR by using *RLFcds*/pENTR as a template with primers H161A-F/H161A-R and H184A-F/H184-R using PrimeSTAR Max DNA Polymerase (Takara Bio Inc.). For the *RLFcds*(H161A/H184A)/pENTR construct, *RLFcds*(H161A)/pENTR was used as a template using H184A-F/H184-R primers with PrimeSTAR Max DNA Polymerase (Takara Bio Inc.). These mutant *RLF*/pENTR were used in the same way to perform LR reactions to construct *MBP-RLF*(H161A), *MBP-RLF*(H184A), and *MBP-RLF*(H161A.H184A). Similarly, an expression clone for MBP-MpRLF was also constructed using the same method by an LR reaction with Mp*RLFcds*/pENTR. The expression clone was transformed into *E. coli* JM109. The growing colonies were precultured in a 5-ml LB liquid medium containing 50 µg ml⁻¹ ampicillin and 0.2% (w/v) glucose at 37°C for 18 h. Of the preculture medium, 3 ml was added to 300 ml of LB liquid medium containing 50 µg ml⁻¹ ampicillin and 0.2% (w/v) glucose at 37°C, 1.31 g for 2–3 h of incubation, followed by shaking at 20°C, 1.31 g for 1 h. Of the 100 mM isopropyl β-D-thiogalactopyranoside (Takara Bio Inc.), 1 ml was added to the culture medium, and the mixture was shaken at 20°C, 0.73 g for 20 h. The cell pellet was collected by centrifugation at 1175 g for 10 min at 4°C, resuspended in 1× PBS, and centrifuged again at 1175 g for 10 min at 4°C. The collected cell pellet was stored at –80°C.

Upon use, the pellet was suspended in 5 ml of Buffer A (20 mM Tris–HCl pH 7.5, 200 mM NaCl) and transferred to a 10-ml beaker. To suppress the activity of proteolytic enzymes, 20 µl of 10 mg ml⁻¹ phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, St. Louis, MO, USA) was added to the bacterial suspension before lysis by sonication. After lysis treatment, the extract was aliquoted into 1.5-ml tubes, centrifuged at 4°C, 16 000 g for 15 min, and the supernatant was collected in 50-ml tubes. Of amylose resin (50% (w/v) slurry), 300 µl was added and allowed to stand at 4°C for 30 min with moderate mixing. The mixture was then centrifuged at 4°C for 1 min at 500 g, and the supernatant was discarded. Subsequently, 1 ml of Buffer A was added to the resin precipitate and collected in a 2-ml tube. After washing, 250 µl of Buffer B (20 mM Tris–HCl pH 9.5, 200 mM NaCl, and 10 mM maltose) was added to the resin, with the supernatant discarded, and allowed to stand at 4°C for 30 min with moderate mixing. After centrifugation at 4°C, 500 g for 1 min, the supernatant was collected, and 6 × Stock buffer (60% (v/v) glycerol, 0.3% (v/v) Triton-100) was added before storing at –80°C.

For measuring the ultraviolet (UV)-visible absorption spectrum of RLF proteins, samples were centrifuged to remove impurities and exchanged by ultrafiltration into a buffer containing 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, and 6% (v/v) glycerol. The protein concentration was adjusted to 1.5 µM. Excess sodium ferricyanide in solution and a few grains of solid dithionite were added to samples to establish the oxidized and reduced heme conditions, respectively. Measurements of the absorbance spectra were performed using a spectrophotometer UV-1900i (Shimadzu, Kyoto, Japan).

RNA-seq and transcriptomic analysis

Total RNA was purified from 16-d-old thalli (c. 100 mg fresh weight) using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany); RNA was quantified using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific). RNA aliquots of 10 µg ml⁻¹ or greater from three biological replicates were used for RNA-Seq analysis. Library preparation and RNA-seq analysis

were contracted to BGI Japan (<https://www.bgi.com/jp/home>), and clean reads were obtained from each library. The resulting reads were mapped using MpTak_v6.1r2 from MARPOLBASE (<https://marchantia.info/>), excluding sequences on the U chromosome from the data. We used HISAT2 (Kim *et al.*, 2015) to align the clean reads to the reference genome and then STRINGTIE (Pertea *et al.*, 2015) to assemble them. Finally, the assembly results of all samples were combined using CUFFMERGE. We used BOWTIE2 (Langmead & Salzberg, 2012) to align clean reads to the reference sequence, and then used RSEM (Li & Dewey, 2011) to calculate the expression of genes and transcripts. Enrichment analysis uses Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) to classify gene function and evaluate gene abundance within a particular functional module. Statistically, a hypergeometric model calculates the *P*-value for each functional module. False discovery rate (FDR) correction is performed to determine significant enrichment; functional modules with an FDR of 0.01 or less are considered significant. The bubble chart and GO hierarchical display are based on the results of GO enrichment analysis using GOATOOLS (Klopfenstein *et al.*, 2018). Each bubble indicates the number of genes associated with the GO term, and the color represents the enrichment score ($-\log_{10}(p_fdr_bh)$). $-\log_{10}(p_fdr_bh)$ is the value resulting from a logarithmic transformation of the value of *p*_{fdr_bh} with a base of 10 and sign inversion. The value *p*_{fdr_bh} is calculated by considering multiple comparisons using the Benjamin–Hochberg method. This method is applied to *p*_{uncorrected}, which indicates whether each GO term is significantly enriched. The GO hierarchy display visualizes the relationship between each GO term using Graphviz on the results of the GO enrichment analysis (Ellson *et al.*, 2002; Klopfenstein *et al.*, 2018). Each GO term includes the log-transformed value of *p*_{uncorrected}. All RNA-Seq data for the Tak-1 and the *Mprlf^{ec}* mutant were deposited in the NCBI Sequence Read Archive (SRA) (PRJNA1225401).

Results

Heme-binding ability is necessary for RLF function in *A. thaliana*

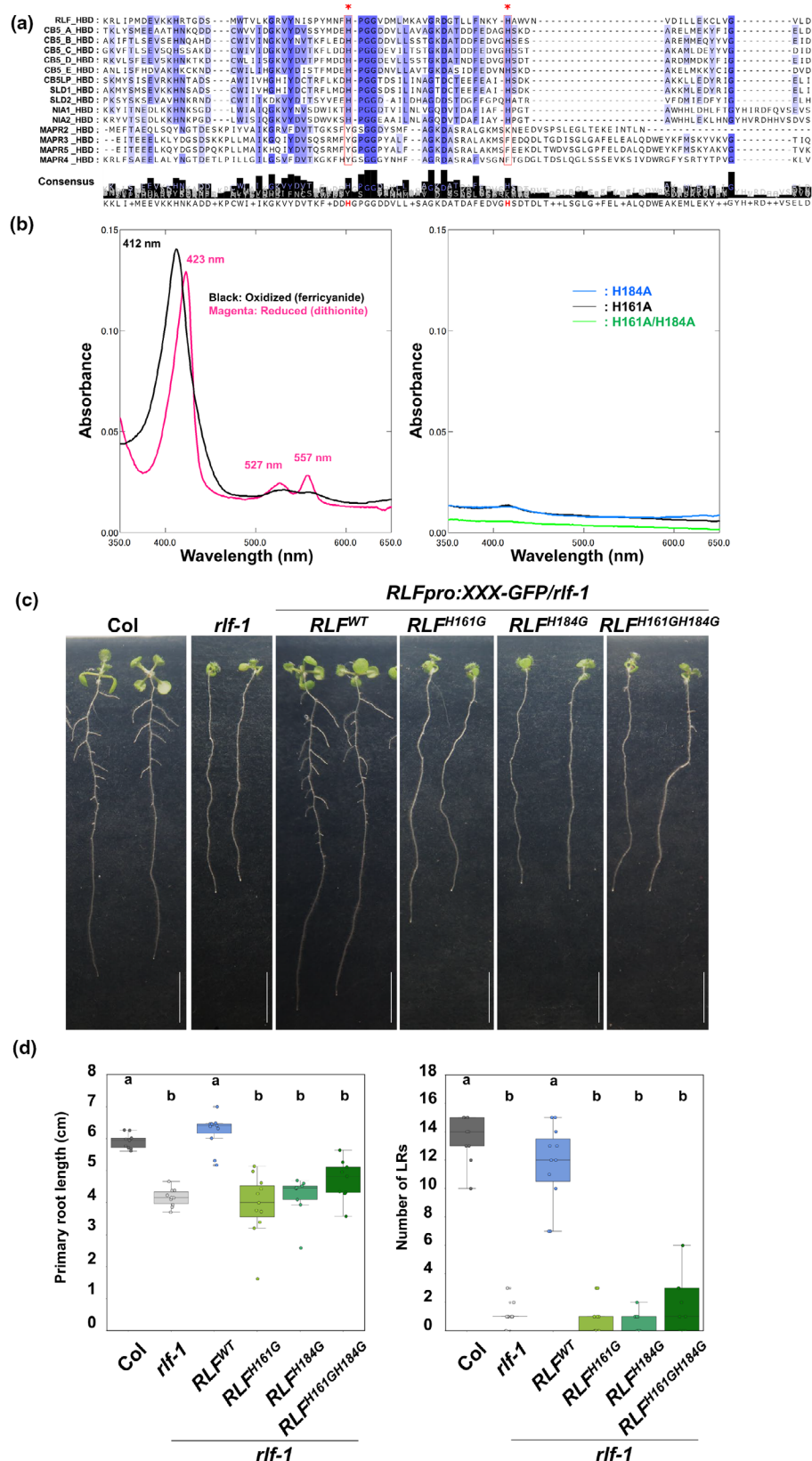
Two histidine residues in Cytb5-HBD are known to be important for heme binding (Cowley *et al.*, 2002; Schenkman & Jansson, 2003). Comparison of the amino acid sequence of the Cytb5-HBD in RLF with those of the other Cytb5-HBD proteins in *A. thaliana* revealed that two histidine residues at positions 161 and 184 of RLF correspond to the predicted heme-binding sites and are conserved among all Cytb5-HBD proteins except for the MAPR family (Figs 1a, S1a). To confirm the importance of these histidine residues, we purified recombinant WT and mutant RLF proteins with individual or combined histidine-to-alanine substitutions (H161A, H184A, and H161A/H184A; Fig. S1b) and measured their absorbance spectra. The pellet of *E. coli* cells expressing WT MBP-RLF appeared reddish brown, indicating the presence of heme, whereas this coloration was not observed in the mutant RLF (Fig. S1c). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis confirmed the expression and

purification of MBP-RLF and mutant MBP-RLF (Fig. S1d). The oxidized form of WT RLF showed a peak in the sorrel band (412 nm) characteristic of Cytb5-HBD proteins. By contrast, the reduced form of RLF showed peaks in the $\alpha\beta$ band (527 and 557 nm) in addition to the sorrel band (423 nm) (Fig. 1b). These absorption spectra of WT RLF are characteristic of Cytb5-HBD proteins (Smith *et al.*, 1994; Wahl *et al.*, 2010). By contrast, the spectra of mutant RLF proteins (H161A, H184A, and H161A/H184A) displayed a striking absence of any of these characteristic peaks, confirming the critical role of histidine residues in heme binding (Fig. 1b). The structure of each mutant RLF protein, predicted using COLABFOLD (Mirdita *et al.*, 2022), showed that the C-terminal CytB5-HBD is highly conserved between WT and mutant proteins (Fig. S1b). This suggests that the heme-binding site substitutions do not affect the overall protein structure. These results indicate that RLF is a HBP and confirm that two histidine residues at positions 161 and 184 are necessary for heme binding to RLF *in vitro*.

Although RLF binds to heme *in vitro*, the functional role of heme binding to RLF *in planta* remains unclear. To address this question, we generated transgenic *A. thaliana* plants expressing the mutant RLF where histidine residues at positions 161 and 184, individually or in combination, were replaced with glycine (H161G, H184G, and H161G/H184G), in the *rlf-1* mutant background (Ikeyama *et al.*, 2010). As shown previously, the *rlf-1* mutant displayed a marked reduction in the number of LRs and reduced primary root growth, compared with WT (Fig. 1c, d). The *RLFpro:RLF-GFP* transgene expressing the GFP-tagged WT RLF successfully rescued the *rlf-1* mutant phenotype (Fig. 1c,d). By contrast, the expression of the GFP-tagged mutant RLF (*RLFpro:RLF(H161G)-GFP*, *RLFpro:RLF(H184G)-GFP*, and *RLFpro:RLF(H161G/H184G)-GFP*) under the control of the *RLF* promoter failed to restore LR formation and primary root growth in the *rlf-1* mutant (Fig. 1c,d). These results indicate that the two histidine residues at positions 161 and 184 are important for RLF function *in planta*, implying that heme binding is necessary for the biological function of RLF.

Conservation of RLF across diverse plant species

A previous study has shown that the *RLF* orthologous genes are present in several plants, including rice (*Oryza sativa*), a monocot, and the moss *Physcomitrium patens* (Ikeyama *et al.*, 2010). To confirm amino acid sequence conservation among RLF orthologs, we first performed multiple alignment comparisons using JALVIEW (Waterhouse *et al.*, 2009) on the amino acid sequences from *A. thaliana* (At5g09680), *Glycine max* (Wm82.a6 Glyma.12G009100, Wm82.a6 Glyma.09G227600), *Oryza sativa* (LOC_Os07g12830), *Amborella trichopoda* (AmTr_v1.0_scaffold00029.68), *Thuja plicata* (Thupl.29380466s0005), *Selaginella moellendorffii* (127 066, 138 045), *M. polymorpha* (Mp3g04390), *Physcomitrium patens* (Pp3c20_23480), *Phaeoceros carolinianus* (RXRQ_scaffold_2017981), *Chlamydomonas reinhardtii* (Cre13_g 574 800), *Volvox carteri* (Vocar.0001-s0218), *Chara braunii* (CBR_g29320), and *Porphyra umbilicalis* (Pum0348s0012). This analysis revealed that the C-terminal



orthologs (Fig. S2a). Particularly, the two histidine residues, which are part of the heme-binding site, are highly conserved in the C-terminal part of the CytB5-HBD region (Fig. S2b). These



Fig. 2 Molecular phylogenetic tree of the REDUCED LATERAL ROOT FORMATION (RLF) protein family. The unrooted molecular phylogenetic tree was constructed based on amino acid sequences of RLF and CB5A, B, C, D, E, and LP from various plant species. The tree includes sequences from *Arabidopsis thaliana* (At5g09680), *Glycine max* (Wm82.a6 Glyma.12G009100 and Glyma.09G227600), *Oryza sativa* (Os07g0232200), *Amborella trichopoda* (AmTr_v1.0_scaffold00029.68), *Thuja plicata* (Thupl.29380466s0005), *Selaginella moellendorffii* (127 066 and 138 045), *Marchantia polymorpha* (Mp3g04390), *Physcomitrium patens* (Pp6c20_12240V6), *Phaeoceros carolinianus* (RXRQ_scaffold_2017981), *Chlamydomonas reinhardtii* (Cre13.g574800), *Volvox carteri* (Voccar.0001s0218), *Chara braunii* (CBR_g29320), and *Porphyra umbilicalis* (Pum0348s0012). Each clade is labeled according to its classification (e.g. RLF clade, CB5A/C clade). The phylogenetic tree was created using the maximum likelihood method (1000 bootstrap tests), the Whelan and Goldman (WAG) model (Whelan & Goldman, 2001), and the MEGA11 software (Tamura *et al.*, 2021). Bootstrap values (1000 tests) are indicated by colors, corresponding to their respective confidence levels, and evolutionary distances are shown below the branches. The molecular phylogenetic tree was constructed using tvBOT (Xie *et al.*, 2023).

findings suggest that the heme binding is crucial for the function of RLF orthologs.

We further constructed a molecular phylogenetic tree that included both RLF orthologs and members of the CB5 family to examine their evolutionary conservation across the plant kingdom (Fig. 2). These analyses revealed that RLF orthologs form a distinct, independent clade that is conserved across a wide variety of plants, including angiosperms, gymnosperms, lycophytes, liverworts, mosses, charophyte algae, and chlorophyta (Table 1). The phylogenetic tree is consistent with previous studies on CB5 phylogeny, such as the grouping of CB5B and CB5D within the same clade, supporting the validity of the tree (Zhao *et al.*, 2024). Furthermore, most plant species have a single RLF ortholog, except for *Glycine max* and *Selaginella moellendorffii* (Table 1). These findings demonstrate that the molecular phylogenetic tree aligns well with previously established classifications while clearly distinguishing the evolutionary uniqueness of the RLF clade.

MpRLF, the RLF ortholog in *M. polymorpha*, complements the *rfl* mutant phenotype in *A. thaliana*

Although RLF orthologs are conserved among plants, we do not know whether the RLF-dependent mechanisms in organ development are also conserved across species. To examine whether these RLF orthologs also regulate organ development, we used the model bryophyte species *M. polymorpha* and named the *M. polymorpha* RLF ortholog MpRLF. We performed alignment analysis using EMBOSS Matcher (https://www.ebi.ac.uk/jdispatcher/psa/emboss_matcher; Madeira *et al.*, 2024) and structural prediction using ALPHAFOLD2 (modified from uniprot; <https://www.uniprot.org>; Skolnick *et al.*, 2021). This revealed that MpRLF and *Arabidopsis* RLF share 51.3% overall sequence similarity, with particularly high conservation in the Cytb5-HBD domain (86.3%), which is critical for heme-binding activity (Fig. 3a). Given the similarity, we tested whether MpRLF exhibits a heme-binding spectrum similar to that of the Cytb5-HBD by performing heme-binding assays using recombinant MpRLF proteins. Similar to *Arabidopsis* RLF, the pellet of *E. coli* cells expressing WT MBP-MpRLF appeared reddish brown, indicating the presence of heme, and SDS-PAGE analysis confirmed the expression and purification of MBP-MpRLF (Fig. S1c,d). The WT MpRLF protein exhibited characteristic heme-binding spectra, consistent with those observed for *A. thaliana* RLF (Fig. 3b). These results confirm that MpRLF possesses conserved heme-binding activity, supporting the hypothesis that this property is preserved across land plant RLFs.

To assess whether MpRLF gene function is conserved between *M. polymorpha* and *A. thaliana*, we expressed MpRLFcds under the control of the RLF promoter in the *A. thaliana* *rfl* mutant background (*RLFpro:MpRLFcds/rfl-1*). As shown in Fig. 3, the expression of MpRLF in the *rfl* mutant fully restored the reduction in LR number and primary root length phenotypes (Fig. 3c, d). These results indicate that MpRLF is functionally interchangeable with *Arabidopsis* RLF, suggesting that the RLF-dependent mechanisms in organ development are conserved and have been independently co-opted in both *A. thaliana* and *M. polymorpha*.

MpRLF is expressed along the midrib of the thallus in *M. polymorpha*

Next, we examined the expression pattern of MpRLF in *M. polymorpha*. The recently published *M. polymorpha* database, Marchantia Expression (<https://marchantia.info/mbex/>; Kawamura *et al.*, 2022), indicates that MpRLF is highly expressed in mature archegoniophores and archegonia (Fig. S3). MpRLF expression was also observed in antheridiophores (which support and elevate antheridia), thalli, gemma cups, and the midrib. By contrast, expression levels were extremely low in gametophyte apical cells and young sporophytes (Fig. S3). These results suggest that MpRLF expression is associated with organ development during both vegetative and reproductive growth phases. To elucidate the expression pattern of MpRLF in detail, we generated transgenic *M. polymorpha* plants expressing the GUS reporter gene under the control of the MpRLF promoter (*MpRLFpro:GUS*). In *MpRLFpro:GUS* lines, GUS activity was detected by histological staining in the central region of gemmae and in the midrib and near the apical meristem of 14-d-old thalli. By contrast, GUS activity was mostly absent in the rhizoid, except for a weak signal observed in one transgenic line (line #2) (Fig. 4a). In transverse and longitudinal sections of the mature thallus, GUS activity was detected along the midrib on the ventral side of the thallus (Fig. 4b,c). Additionally, histological observations of gemma cups revealed GUS staining in both the gemmae and the base of the gemma cups (Fig. 4d). These results indicate that MpRLF is expressed in the thallus and during gemma formation. Furthermore, in antheridiophores, GUS activity was observed in the antheridial disks and parts of the stalk (Fig. 4e). These observations are consistent with the publicly available expression data from Fig. S3, further supporting that MpRLF is expressed in various organs during both vegetative and reproductive growth phases.

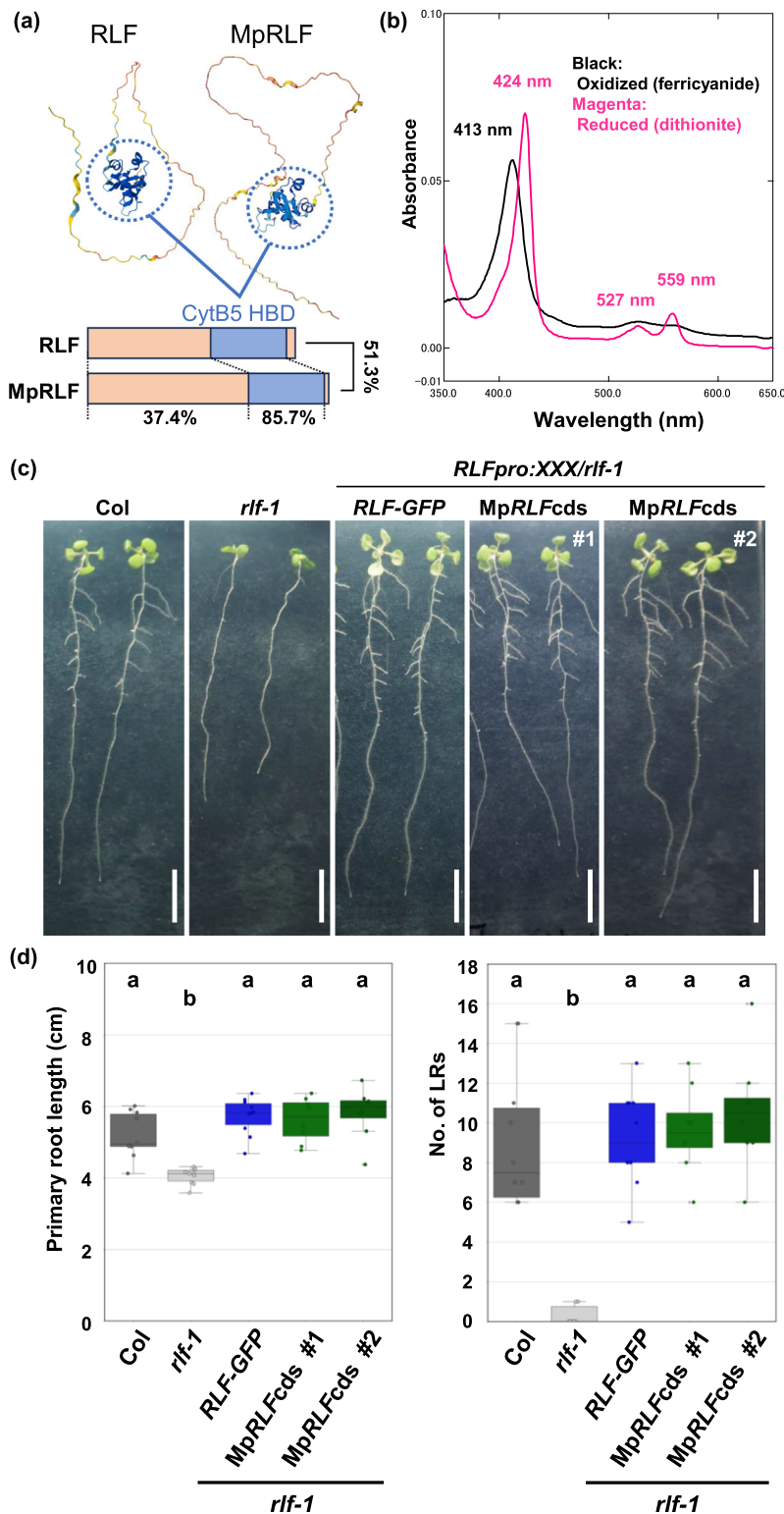


Fig. 3 *MpRLF* rescues the *rlf* mutant phenotype in *Arabidopsis thaliana*. (a) Structure of REDUCED LATERAL ROOT FORMATION (RLF) and *MpRLF* as predicted by ALPHAFOLD2. The numbers indicate amino acid sequence similarity: overall 51.3%, N-terminal region 37.4%, and CytB5 HBD 85.7%. Sequence alignment was performed using EMBOSS Matcher (https://www.ebi.ac.uk/jdispatcher/psa/emboss_matcher; Madeira *et al.*, 2024). (b) Absorbance spectra of wild-type (WT) maltose-binding protein (MBP)-*MpRLF* proteins under oxidized and reduced conditions. Oxidation was induced using ferricyanide, while reduction was achieved with dithionite. The measurement method was performed as described by Takahashi *et al.* (2008). (c) Nine-day-old *A. thaliana* seedlings of WT, *rlf-1* mutant, and *rlf-1* mutants expressing *RLFpro::RLFcds-GFP* and *RLFpro::MpRLFcds*. Bars, 1 cm. (d) Primary root length and number of lateral roots (LRs) in 9 d-old *A. thaliana* WT (Columbia (Col-0)), *rlf-1*, *RLFpro::RLF-GFP/rlf-1*, and *RLFpro::MpRLFcds/rlf-1* seedlings. Primary root length and number of LR were measured for Col-0 ($n = 10$), *rlf-1* ($n = 10$), *RLFpro::RLF-GFP/rlf-1* ($n = 10$), *RLFpro::MpRLF/rlf-1* #1 ($n = 8$), and *RLFpro::MpRLF/rlf-1* #2 ($n = 8$). The band near the middle of the boxplot represents the median. The box indicates the interquartile range, and the whiskers extend to the smallest and largest values within 1.5 times the interquartile range. Samples denoted by different letters are significantly different ($P < 0.05$, the Tukey–Kramer test).

MpRLF protein is localized to the cytosol

In *A. thaliana*, RLF localizes to the cytosol (Ikeyama *et al.*, 2010). To investigate the subcellular localization of *MpRLF*, the *MpE2Fpro::XVE>MpRLFcds-GFP* lines described above were

used for observation. In the epidermal cells near the apical notch of the *MpE2Fpro::XVE>MpRLFcds-GFP* gemmaling grown for 24 h on an EST-containing medium, *MpRLF-GFP* was localized to the cytosol and did not co-localize with chloroplasts, the nucleus, or the plasma membrane (Fig. 4f), which is consistent

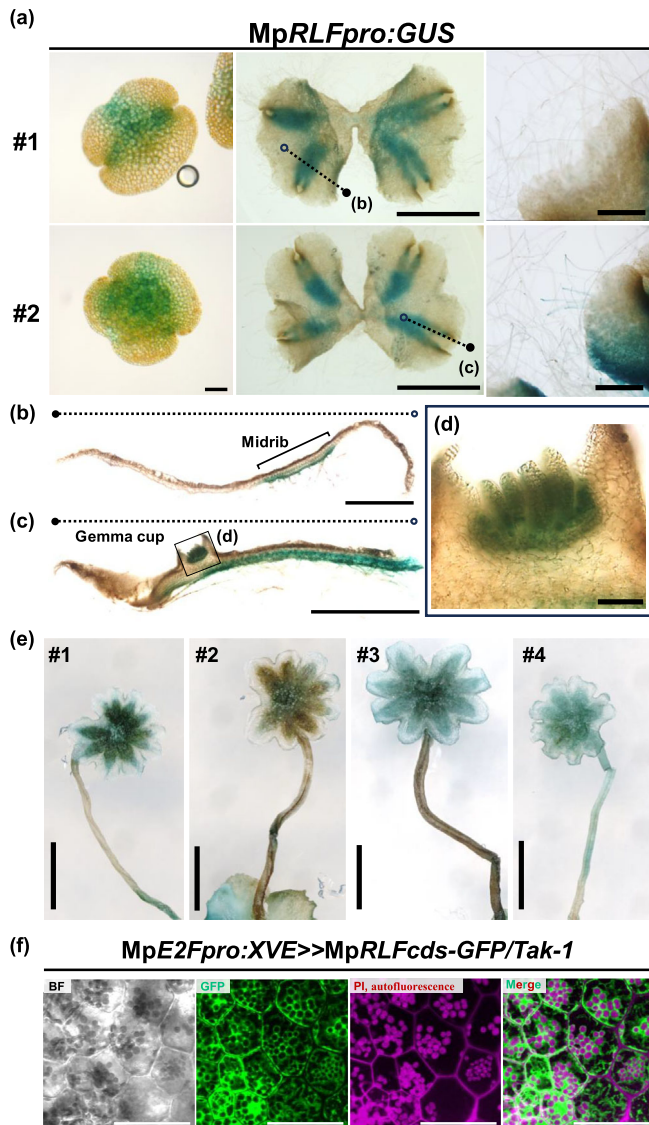


Fig. 4 Expression pattern of MpRLF in *Marchantia polymorpha*. (a) β -Glucuronidase (GUS) staining of MpRLFpro:GUS reporter lines. GUS activity in gemmae after 24 h of staining (left panel), in 14-d-old thalli stained for 4 h (middle panel) and in 14-d-old rhizoids stained for 4 h (right panel). Bars: left, 100 μ m; middle, 5 mm; right, 1 mm. Dashed lines indicate the directions of cross sections shown in (b) and (c). (b, c) Expression pattern of MpRLFpro:GUS in cross sections of thalli. (b) Transverse section showing GUS activity in the midrib area. (c) Longitudinal section showing GUS activity near the base of the gemma cups shown in (d). Bars, 1 mm. (d) GUS activity in developing gemmae and the gemma cups. Bars, 100 μ m. (e) GUS staining for 4 h in reproductive organs reveals GUS activity in male reproductive organs. Bars, 5 mm. (f) Cellular localization of the MpRLF protein. Mp2Fpro: XVE>>MpRLFcds-GFP/Takaragake-1 (Tak-1) gemmae were treated with 10 μ M estradiol for 24 h. Green indicates GFP fluorescence (MpRLFcds-GFP), and magenta indicates chloroplast autofluorescence and propidium iodide (PI) fluorescence. Bars, 50 μ m.

with the subcellular localization of the *Arabidopsis* RLF (RLF-GFP) protein (Ikeyama *et al.*, 2010). These results suggest that MpRLF functions in the cytosol.

MpRLF is essential for proper thallus and gemma cup formation

To investigate the biological role of MpRLF, we produced a mutant of MpRLF (*Mprlf^{fe}*) using the CRISPR-Cas9 method. The target sequence of CRISPR-Cas9 was designed at the junction of the second intron and the third exon, upstream of the Cytb5-HBD (Fig. 5a). Sequencing analysis of the obtained mutants revealed deletions of -2, -18, and -1 bp in the genomic region near the target site in independent lines (*Mprlf^{fe}* #3, #9, and #11), respectively (Fig. 5b). In addition, these deletions caused frameshifts in these mRNA sequences, causing premature stop codons to occur upstream of the Cytb5-HBD (Fig. 5c). All three alleles of the *Mprlf^{fe}* mutant showed growth retardation compared with WT (Figs 5d,e, S4a). The WT plant formed the gemma cups with gemmae on the dorsal surface of the thallus, while in all three *Mprlf^{fe}* mutants, the formation of proper gemma cups was inhibited and gemma-like structures formed directly on the thallus surface (Fig. 6a). A complementation test with an MpRLF coding sequence with synonymous CRISPR-resistant mutations (*MpRLFcds_resistant*; Fig. S4b) showed that transgenic plants expressing MpRLFcds_resistant under the control of the MpRLF promoter in an *Mprlf^{fe}* background grew as well as WT and formed proper gemma cups (Fig. 5d,e). These results indicate that phenotypes observed in *Mprlf^{fe}* (retardation of thallus growth and impaired gemma cup formation) are due to the loss-of-function mutation in the MpRLF gene, thereby confirming that MpRLF is essential for proper thallus growth and gemma cup formation.

Furthermore, a comparison of rhizoid elongation among WT, mutants, and complementation lines revealed that *Mprlf^{fe}* mutants exhibited a slightly shorter overall rhizoid length (Fig. 6b,c). However, some individuals were observed to possess rhizoids comparable in length to those of WT, and no significant abnormalities in rhizoid development were detected. These results, together with the weak GUS expression observed in rhizoids, suggest that MpRLF may play a minor role in rhizoid formation.

Next, we induced reproductive organ formation under far-red light and white light and observed the development of antheridiophores. In WT and complementation lines, antheridiophores containing antheridia were formed, whereas in *Mprlf^{fe}* mutants, antheridiophores appeared shorter, and the shape of the antheridial disk was altered (Fig. 7a,b). Analyses of the antheridial disk size, stalk length, and stalk diameter showed that the disk size in the mutants matched WT, but the stalks were shorter and thicker (Fig. 7c-e). Additionally, we observed that in *Mprlf^{fe}*, antheridium-like structures were exposed on the surface of the antheridial disk, whereas in WT plants, these structures are normally enclosed within the disk (Fig. 7f,g). In addition, we generated several *Mprlf^{fe}* mutant lines in the female Tak-2 background using the CRISPR-Cas9 method (Fig. S4c). When reproductive organs were induced in these mutants, archegoniophores were shorter in height than WT and formed irregular archegonial disks lacking normal protrusions (Fig. 8a,b). Measurements of the archegonial disk area, stalk length, and stalk diameter revealed

that the mutants had a smaller archegonial disk area and longer stalks than WT, while no significant difference was observed in stalk diameter (Fig. 8c–e). These abnormalities in the

reproductive organs of male and female plants were observed even at early stages of reproductive organ development (Fig. S4d). Furthermore, transverse sections of archegoniophores

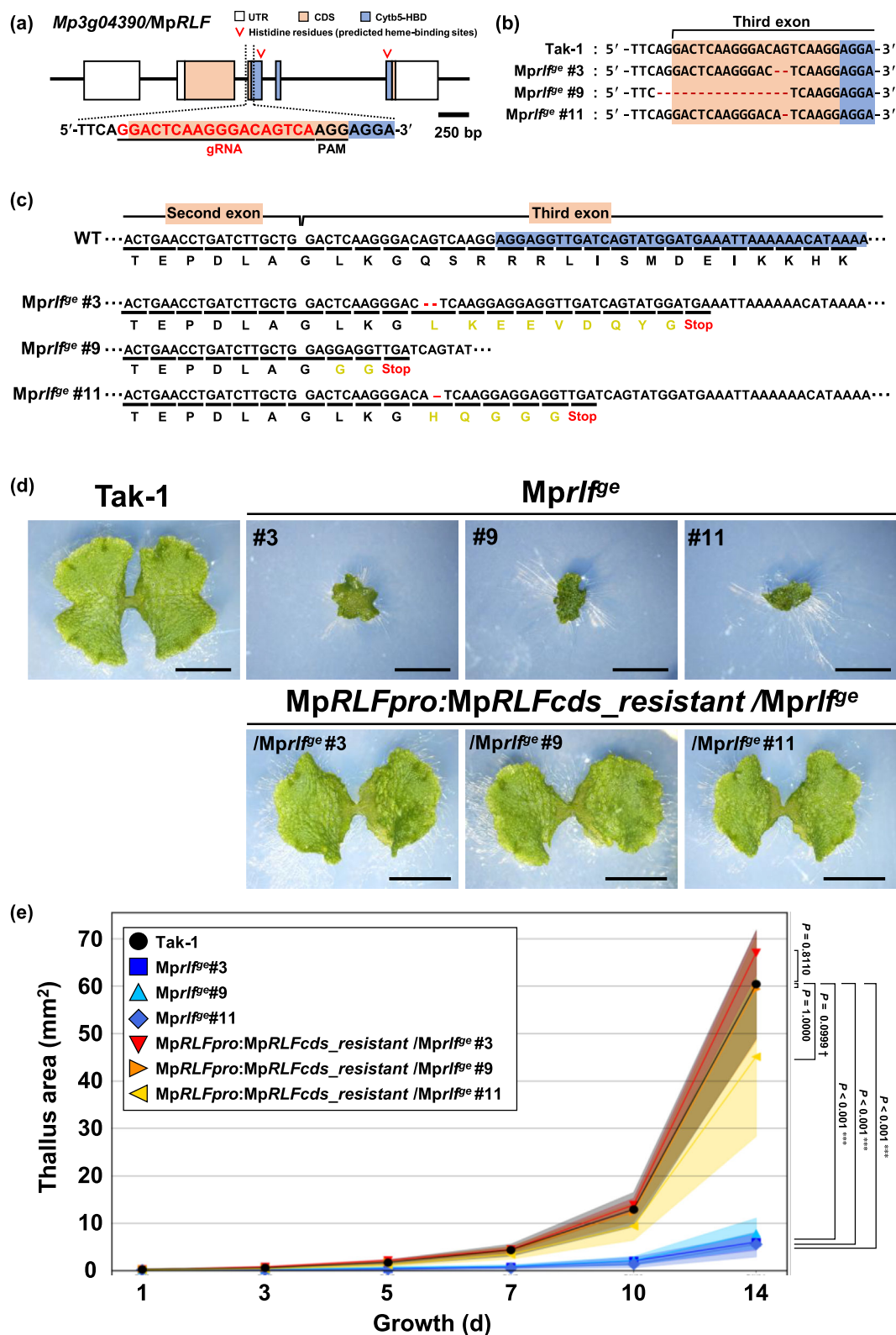
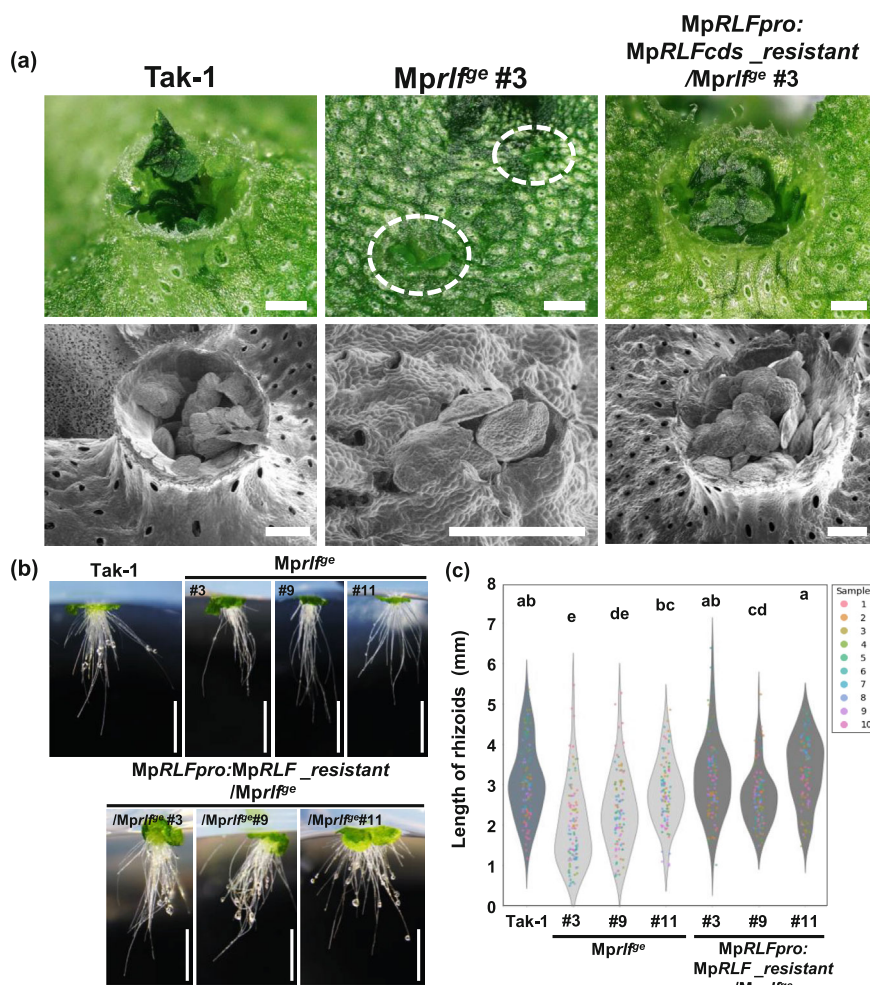


Fig. 5 Mutations in *MpRLF* inhibit vegetative growth and development in *Marchantia polymorpha*. (a) *Mp3g04390/MpRLF* gene structure and selected target sequence for CRISPR mutagenesis. White, orange, and blue boxes indicate exons in the untranslated region, protein coding region, and cytochrome *b₅*-like heme-binding domain (Cytb5-HBD), respectively. The underlined sequence is the CRISPR's target sequence; red bases indicate gRNA sequence, and black bases indicate Protospacer Adjacent Motif (PAM) sequences. (b) Sanger sequencing analysis of mutations at gRNA target site. Dashed lines indicate deleted bases. (c) The mutations in the *MpRLF* coding sequence (from the second to third exon) and the corresponding amino acid sequences are shown. Premature stop codons resulting from frame-shift mutations are indicated in red. Altered amino acid sequences resulting from frame-shift mutations are indicated in yellow. The coding sequence highlighted in blue corresponds to the Cytb5-HBD. (d) Wild-type (WT) *M. polymorpha* (Takaragaike-1 (Tak-1)), *Mprl^{fse}* mutants, and complementation lines (*MpRLFpro:MpRLFcds_resistant* in *Mprl^{fse}* mutants) grown for 10 d. Bars, 5 mm. (e) Thallus growth area 2 wk after gemma germination. Data are presented as means, and shaded areas represent 95% confidence intervals. Takaragaike-1 (Tak-1) (*n* = 9), *Mprl^{fse}* #3 (*n* = 8), *Mprl^{fse}* #9 (*n* = 10), *Mprl^{fse}* #11 (*n* = 10), *MpRLFpro:MpRLFcds_resistant/Mprl^{fse}* #3 (*n* = 10), *MpRLFpro:MpRLFcds_resistant/Mprl^{fse}* #9 (*n* = 9), and *MpRLFpro:MpRLFcds_resistant/Mprl^{fse}* #11 (*n* = 10) were used for analysis. Statistical analysis was performed using Dunnett's test on d 14, using WT as the control, with adjusted *P*-values calculated using the single-step method. Significance levels were set as follows: ***, *P* < 0.001; and †, *P* < 0.1.

Fig. 6 Mutations in *MpRLF* inhibit the formation of gemma cups and reproductive organs. (a) Phenotypes of the gemma cups on the dorsal surface of thalli in wild-type (WT) (Takaragaike-1 (Tak-1)), *Mprl^{fse}* #3 mutants, and the complementation line (*MpRLFpro:MpRLFcds_resistant/Mprl^{fse}* #3) grown for 3 wk. The top row shows brightfield images, while the bottom row shows scanning electron microscope (SEM) images. Bars, 500 μ m. (b) Representative images and quantitative analysis of rhizoid length in Tak-1, *Mprl^{fse}* mutants (#3, #9, and #11), and complementation lines (*MpRLFpro:MpRLFcds_resistant/Mprl^{fse}* #3, #9, and #11) after 6 d of growth on agar medium. For the observation of rhizoid growth, the plants were cultivated in an inverted orientation, with the medium placed on the upper side. Bars, 2 mm. (c) Violin plots of rhizoid length. Each point represents the length of individual rhizoids measured from 10 rhizoids per plant across 10 plants per line. Points are color-coded to indicate data from the same individual. The width of the violin plots depicts the density of data points. Statistical groups are labeled (*P* < 0.05, the Tukey–Kramer test).



revealed that the mutants formed abnormal, fully enclosed archegonium-like structures instead of typical archegonia seen in WT (Fig. 8f–j). These results suggest that *MpRLF* regulates multiple aspects of reproductive organ development, including stalk growth and structural proportions.

To investigate the impact of *MpRLF* overexpression on organ development, we generated transgenic plants with *MpRLFcds*-

GFP expression inducible by EST under the control of *MpE2Fpro* (*MpE2Fpro:XVE* > *MpRLFcds-GFP*). Interestingly, while the loss-of-function mutants exhibited reduced thallus area, EST-induced overexpression of *MpRLF* (*MpRLF-GFP*) clearly suppressed thallus growth (Fig. S5). These results suggest that proper regulation of *MpRLF* expression is important for the vegetative growth of *M. polymorpha*. Taken together, these results

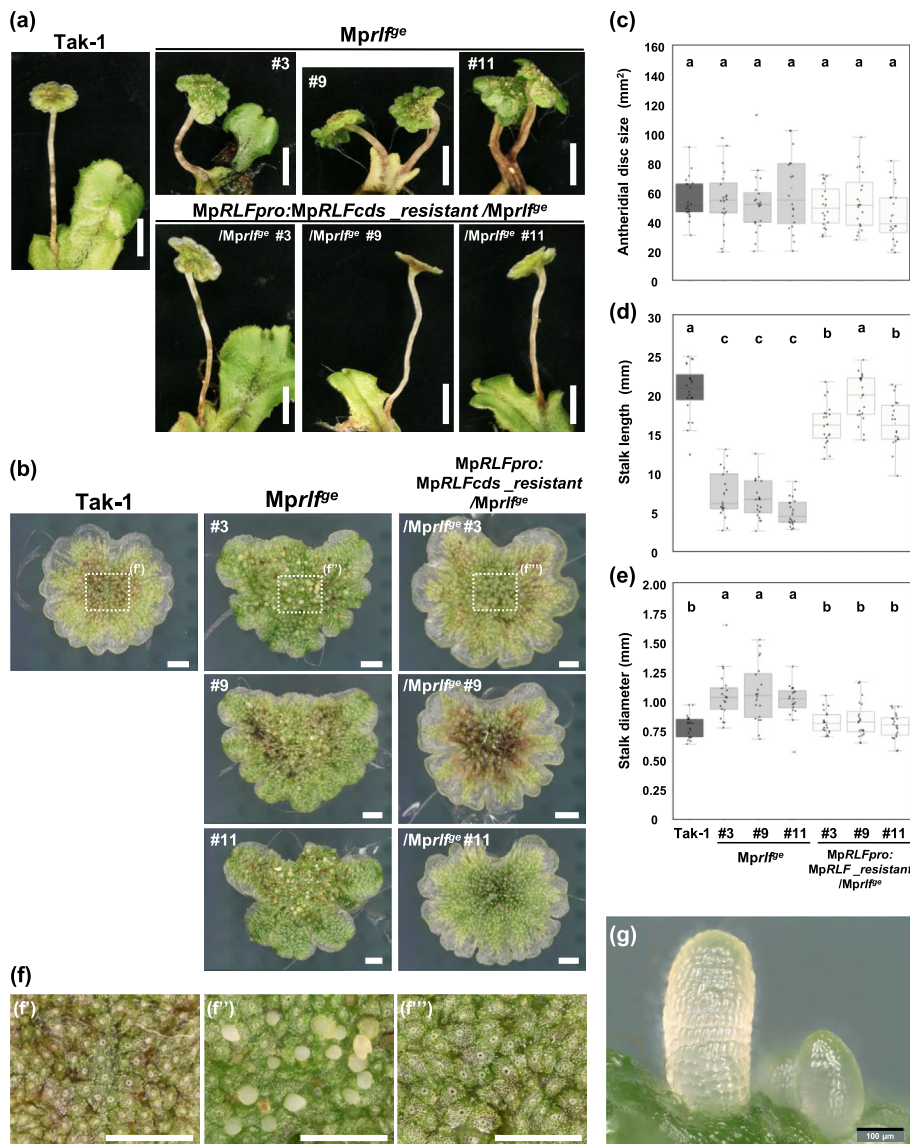


Fig. 7 Developmental defects in antheridiophore formation of *Mprlf^{se}* mutants. Reproductive organs of *Mprlf^{se}* mutants were examined under light conditions conducive to reproductive growth (3 wk under white light followed by 6 wk under far-red light and white light). (a) Phenotype of antheridiophores in wild-type (WT) (Takaraige-1 (Tak-1)) and *Mprlf^{se}* mutants. Bars, 5 mm. (b) Morphology of the antheridial disks in WT and *Mprlf^{se}* mutant plants, and *MpRLFpro:MpRLFcds_resistant* complementation lines. Bars, 1 mm. (c–e) Quantitative measurements for antheridial disk area (c), stalk length (d), and stalk diameter (e). The band near the middle of the boxplot represents the median. The box indicates the interquartile range, and the whiskers extend to the smallest and largest values within 1.5 times the interquartile range. Statistical analysis shows significant differences among genotypes, with letters indicating statistically distinct groups ($n = 20$, $P < 0.05$, the Tukey–Kramer test). (f) Close-up views of the areas marked in (b). Bars, 1 mm. (g) Close-up images of the aberrant antheridium-like structure observed in *Mprlf^{se}* mutants. Bar, 100 μ m.

indicate that *MpRLF* is required for proper organ formation during the vegetative and reproductive growth of *M. polymorpha*.

Arabidopsis thaliana RLF partially complements the *Mprlf^{se}* mutant phenotype in *M. polymorpha*

To examine the functional conservation of RLF between *A. thaliana* and *M. polymorpha*, we conducted a complementation test using the *Mprlf^{se}* #3 mutant and found that lines expressing *A. thaliana* RLF under the *MpRLF* promoter (*MpRLFpro:AtRLFcds/Mprlf^{se}* #3) exhibited partial rescue of the mutant phenotype in thallus morphology and gemma cup formation (Fig. 9). These results and the fact that *MpRLF* rescues the LR formation phenotype of the *A. thaliana* *rlf* mutant (Fig. 3c,d) indicate that *MpRLF* and *A. thaliana* RLF share conserved and common molecular functions that are critical for organ development.

MpRLF strongly alters expression of metabolism-related genes

Finally, to gain insight into the pathways through which *MpRLF* regulates organ growth and development, we performed RNA sequencing (RNA-Seq) analysis using Tak-1 and the *Mprlf^{se}* mutant (*Mprlf^{se}* #3). Of the sequences obtained from the clean reads, 97.96% could be mapped to the *M. polymorpha* genome (Marpolbase MpTak_v6.1r2; Bowman *et al.*, 2017; Montgomery *et al.*, 2020). There are 3734 differentially expressed genes (DEGs) in *Mprlf^{se}*, with 1839 upregulated and 1895 downregulated (Fig. 10a).

To better characterize the gene expression variations in *Mprlf^{se}*, we performed GO enrichment analyses. The results indicate that the top-affected biological processes in *Mprlf^{se}* are cellular processes (GO:0009987) and metabolic processes (GO:0008152). In cellular components, the top-affected

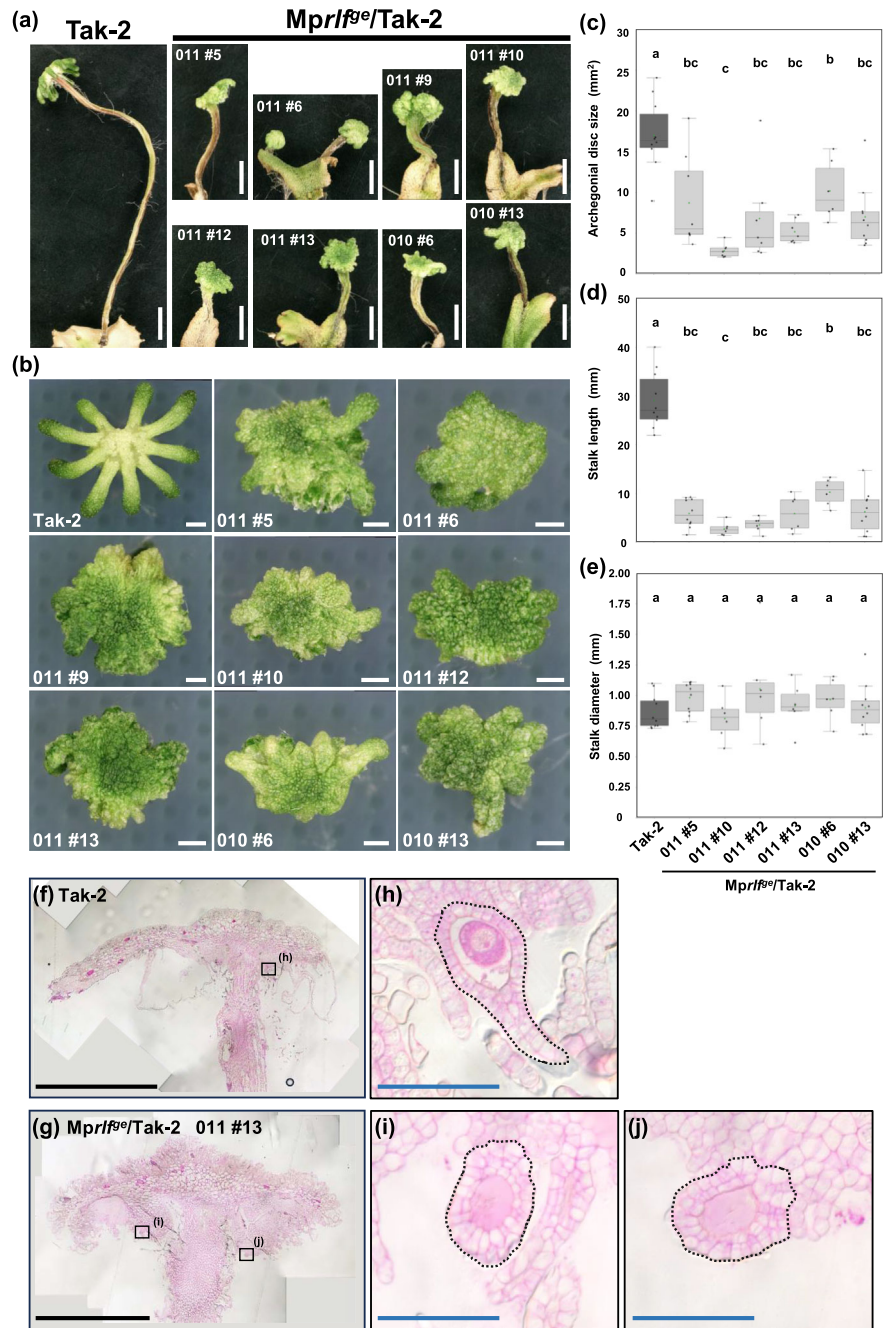


Fig. 8 Observations of archegoniophore formation in *Mprlf^{ge}* mutants. Reproductive organ formation in wild-type (WT) (Takaraikai-2 (Tak-2)) and *Mprlf^{ge}/Tak-2* mutants was induced by incubating gemmae under white light for 3 wk followed by far-red light and white light for 8 wk. The sequence information for each mutant is shown in Supporting Information Fig. S4(c). (a) Representative phenotypes of archegoniophores in WT (Tak-2) and *Mprlf^{ge}/Tak-2* mutants. Bars, 5 mm. (b) Images of archegonial disks in WT (Tak-2) and *Mprlf^{ge}/Tak-2* mutants. Bars, 1 mm. (c–e) Quantitative analysis of archegonial disk area (c), stalk length (d), and stalk diameter (e) was conducted for Tak-2, 011 #5, and 010 #13 ($n = 10$), as well as 011 #10, #12, #13, and 010 #6 ($n = 6$). The band near the middle of the boxplot represents the median. The box indicates the interquartile range, and the whiskers extend to the smallest and largest values within 1.5 times the interquartile range. Statistical significance was determined using the Tukey–Kramer test ($P < 0.05$), with different letters indicating significant differences. (f, g) Cross-sectional images of archegonial disks in WT (f) and *Mprlf^{ge}/Tak-2* mutants (g). Bar, 1 mm. (h–j) Magnified views of the sections shown in (f, g), illustrating the structure of archegonia in WT (Tak-2) (h) and the aberrant archegonium-like structures in mutants (i, j). The archegonia and archegonium-like structures are indicated by black dotted outlines. Bars, 100 μ m.

processes are cell parts (GO:0044464), cell (GO:0005623), membrane (GO:0016020), membrane parts (GO:0044425), organelle (GO:0043226), and organelle part (GO:0044446). In molecular function, binding (GO:0005488) and catalytic activity (GO:0003824) are the top-induced processes (Fig. S6a). Furthermore, KEGG pathway analysis of DEGs showed they are enriched for metabolism-related terms (Fig. S6b). In addition, gene set enrichment analysis (GSEA) using bubble charts showed that, among biological processes, those related to metabolic processes – such as nucleic acid metabolic processes (GO:0090304), secondary metabolic process (GO:0019748), and carboxylic acid metabolic process (GO:0019752) – are enriched in upregulated

genes (Fig. 10b). In the same analysis for the downregulated genes, the cellular nitrogen compound metabolic process (GO:0034641) is particularly enriched. These findings suggest that the metabolic state is drastically altered in *Mprlf^{ge}* (Fig. 10c). Gene set enrichment analysis, based on molecular function, showed that GO terms related to oxidoreduction, such as oxidoreductase activity (GO:0016491) and catechol oxidase activity (GO:0004097), are enriched in upregulated genes, and transmembrane transporter activity (GO:0022857) and heme binding (GO:0020037) are enriched in downregulated genes (Fig. S7a). Gene set enrichment analysis, based on cellular components, revealed that upregulated genes are enriched in apoplast

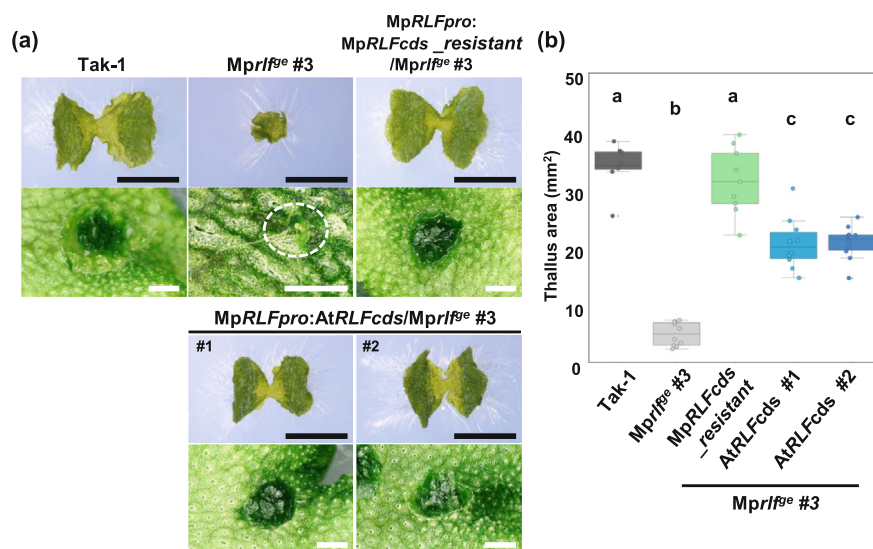


Fig. 9 *Arabidopsis thaliana* RLF partially complements *Mprlf^{se}* mutants in *Marchantia polymorpha*. (a) Representative images of thalli and gemma cups. The top row shows 10-d-old thalli of Takaragake-1 (Tak-1), *Mprlf^{se}* #3, *MpRLFpro: MpRLFcds_resistant/Mprlf^{se}* #3, and *MpRLFpro: AtRLFcds/Mprlf^{se}* #3 (#1 and #2 lines). Bars, 5 mm. The bottom row shows 3-wk-old gemma cups of the same genotypes. The dashed circle highlights the abnormal gemma cup structure in *Mprlf^{se}* #3. Bars, 1 mm. (b) Quantification of thallus area in 10-d-old plants. Boxplots show the thallus area of Tak-1 ($n = 8$), *Mprlf^{se}* #3 ($n = 10$), *MpRLFpro: MpRLFcds_resistant/Mprlf^{se}* #3 ($n = 9$), *MpRLFpro: AtRLFcds/Mprlf^{se}* #3 (#1: $n = 10$, #2: $n = 10$). Statistical analysis was performed using the Tukey–Kramer test ($P < 0.05$), with different letters indicating significant differences. RLF, REDUCED LATERAL ROOT FORMATION.

(GO:0048046) and nuclear protein-containing complex (GO:0140513), while downregulated genes are enriched in protein-containing complex (GO:0032991) and plasma membrane (GO:0005886) (Fig. S7b). In addition, we performed GO hierarchical analysis of biological processes on the top 200 upregulated and downregulated genes. The upregulated genes are enriched for GO terms related to chitin metabolism (Fig. 11a; Table S2). For downregulated genes, GO terms related to nitrate metabolism, transport, and response are enriched (Fig. 11b; Table S2). These results suggest that the aberrant organ growth and development observed in *Mprlf^{se}* might be caused by changes in the metabolic status, potentially involving MpRLF as a Cytb5-HBD protein.

Discussion

In this study, we demonstrated that *A. thaliana* RLF is a HBP and confirmed that two histidine residues at positions 161 and 184 within the Cytb5-HBD are necessary for heme binding *in vitro* and for RLF function *in planta*. RLF orthologs are highly conserved in various green plants, and the two histidine residues, essential for heme binding within the Cytb5-HBD, are found in RLF orthologs. These results imply that RLF orthologs in other plants also bind to heme via two histidine residues and that the heme binding ability of the Cytb5-HBD is necessary for the biological function of RLF proteins. Furthermore, we showed that (1) MpRLF, a RLF ortholog in *M. polymorpha*, also binds to heme *in vitro*, that (2) MpRLF rescues the LR formation mutant phenotype of the *A. thaliana* *rlf* mutant, and that (3) MpRLF is crucial for proper organ development in *M. polymorpha*. These results suggest that RLF orthologs in other plants may also play a role in organ development. Therefore, it will be interesting to verify whether RLF orthologs from various plants and algae, including charophytes, bryophytes, lycophytes, gymnosperms, angiosperms, as well as green and red algae, are able to complement either the *rlf* mutant in *A. thaliana* or the *Mprlf^{se}* mutant in *M. polymorpha*, or both. This would provide insights into the

evolutionary conservation of RLF function across plants and algae.

Transcriptome analysis using the *Mprlf^{se}* mutant also suggests that MpRLF may contribute to organ development by regulating several metabolic pathways. Gene Ontology enrichment and KEGG pathway analyses revealed that the variation in gene expression in *Mprlf^{se}* is most pronounced in metabolic processes, cellular components, and molecular functions. Furthermore, the GO hierarchy of the top 200 downregulated genes was enriched in GO terms related to nitrate response, transport, and metabolism (Fig. 11b; Table S2). Of particular interest is the fact that RLF regulates LR formation in *A. thaliana* (Ikeyama *et al.*, 2010). The link between LR formation and nitrate has been extensively studied in recent years. The nitrate-responsive OBP4-XTH9 regulatory module plays a crucial role in LR development and regulates LR growth in response to changes in nitrate concentration (Xu & Cai, 2019). Furthermore, it has been demonstrated that nitrate interacts with auxin during LR formation, and that the distribution of nitrate concentration significantly affects LR development (Pélissier *et al.*, 2021). We have not examined the involvement of nitrate in RLF-dependent organ development, but there may be a causative link between nitrate and abnormal organ development in *Mprlf^{se}*.

Regarding the *A. thaliana* Cytb5-HBD family, Cytb5-HBD proteins function primarily as an electron carrier, transferring electrons from reductases to target proteins. Specifically, CB5D provides electrons to enzymes that convert precursors of G-lignin synthesis to precursors of S-lignin synthesis, thereby regulating lignin synthesis (Gou *et al.*, 2019). CB5B is involved in sphingolipid synthesis through its interaction with FAH1, which hydroxylates the C-2 position of fatty acids and provides the necessary electrons for the reaction (Nagano *et al.*, 2012). These findings suggest that RLFs may also function as electron transmitters. Consistent with this, our RNA-seq analysis showed that the expression of genes involved in electron transfer and redox is altered in *Mprlf^{se}*, suggesting that MpRLF is an electron transferase that regulates the cellular redox state. Additionally, the

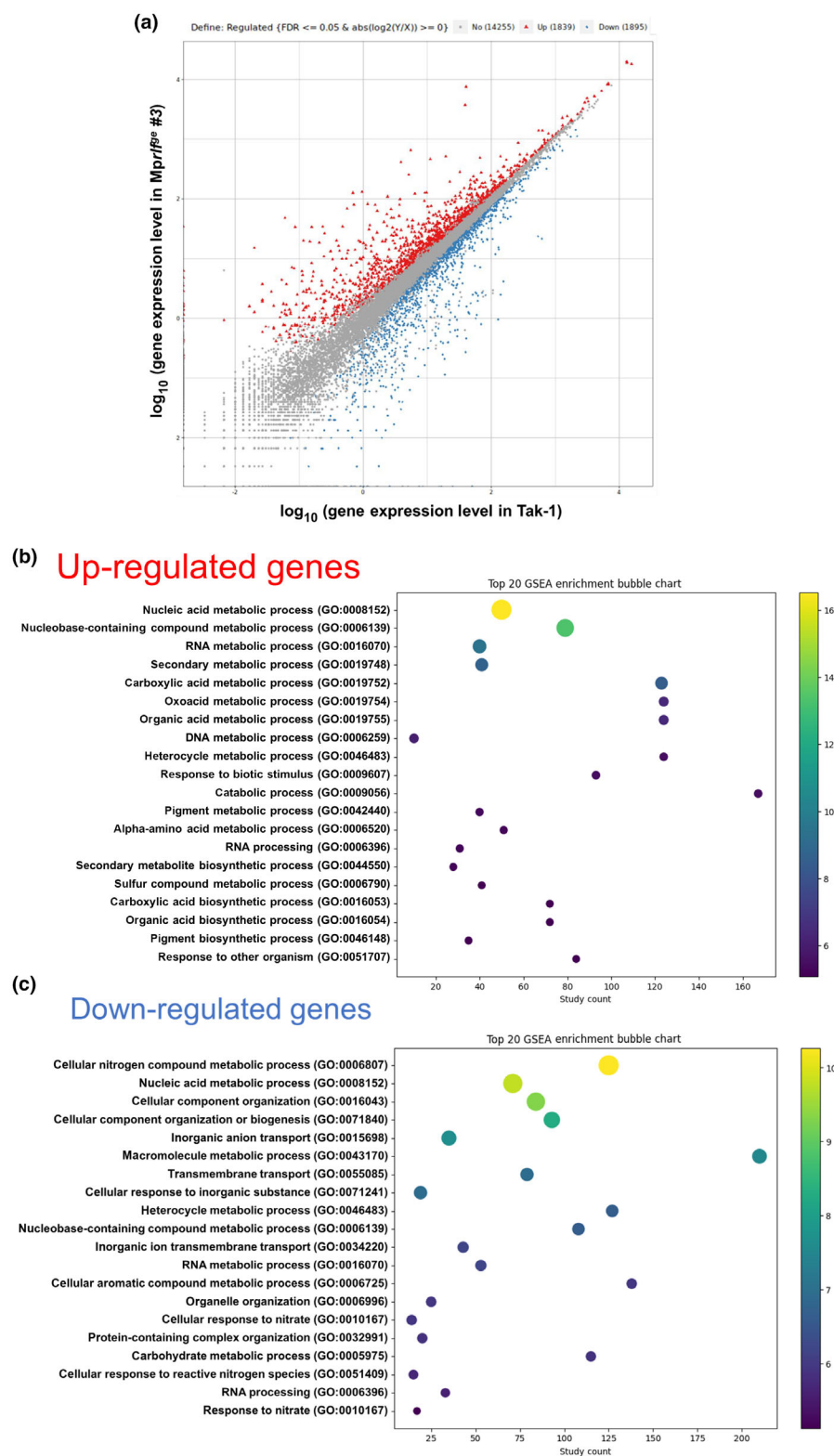


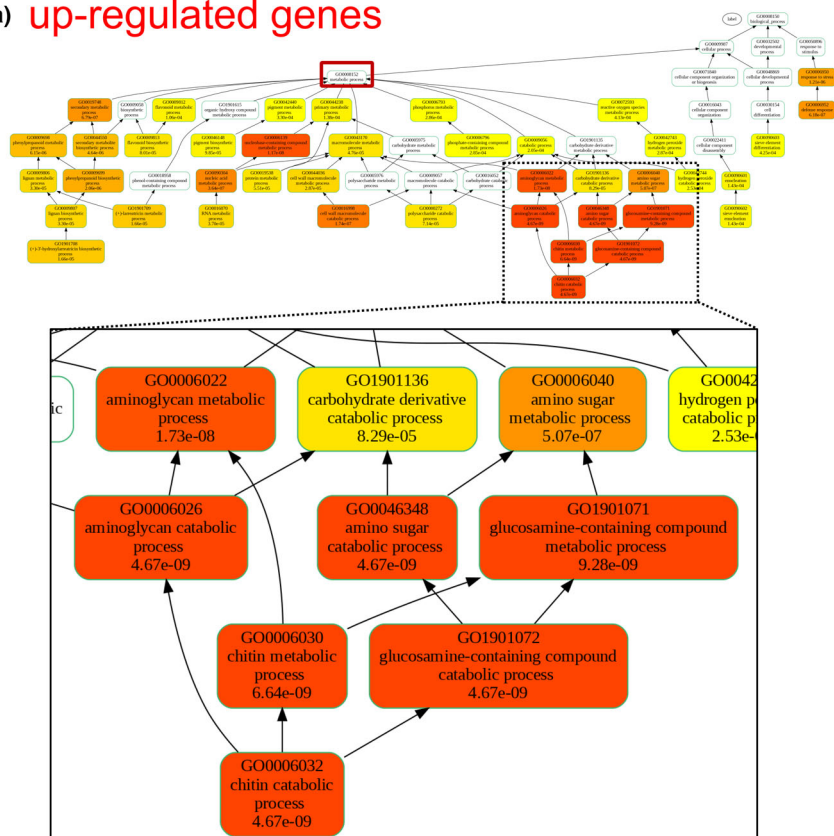
Fig. 10 Transcriptome analysis with MprLF loss-of-function mutants. (a) MA plot of wild-type (WT) and the MprLF^{fe} #3 mutant. Each dot represents the log₁₀-fold change in a gene, with red and blue dots representing up- and downregulated genes in MprLF^{fe} #3 compared with WT, respectively. (b, c) Gene set enrichment analysis (GSEA) based on biological processes in the Gene Ontology (GO) terms. Each bubble chart displays the top 20 GO terms, showing log₁₀ (p_{fdr_bh}) resulting from GO enrichment analysis for the upregulated (b) and downregulated (c) genes in MprLF^{fe} #3. The horizontal axis represents the number of genes, while the color bar represents log₁₀ (p_{fdr_bh}). The value -log₁₀ (p_{fdr_bh}) is obtained by converting the p_{fdr_bh} value to its negative logarithm base 10. FDR, false discovery rate.

Cytb5-HBD protein may also be an electron transferase in *M. polymorpha*, although its specific action pathway remains to be elucidated. Therefore, identifying MprLF-interacting proteins through co-immunoprecipitation or proximity labeling using

active biotin ligase (TurboID) would be valuable (Mair *et al.*, 2019; Zhang *et al.*, 2020).

The MprLF^{fe} mutant exhibited the inhibition of both gemma cup formation and thallus growth, which is consistent with

(a) up-regulated genes



(b) down-regulated genes

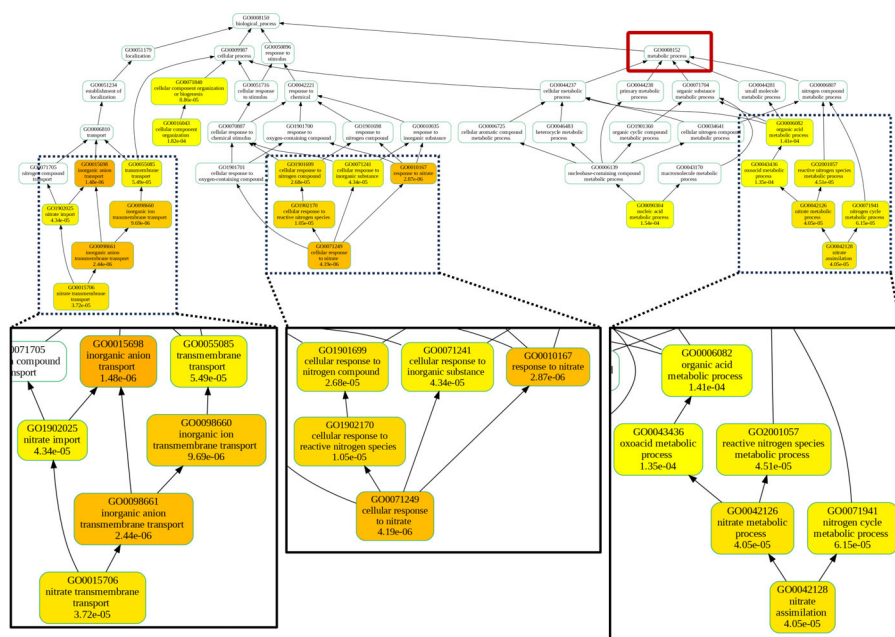


Fig. 11 Gene Ontology (GO) hierarchy of top 200 upregulated and downregulated genes. GO hierarchical structure based on biological processes for the top 200 genes with upregulated expression (a) and the top 200 genes with downregulated expression (b), respectively. Red boxes indicate metabolic process (GO0008152). The GO hierarchy was created by referring to Ellson *et al.* (2002) and Klopfenstein *et al.* (2018).

MpRFL promoter activity at the bottom of the gemma cup and near the meristem of the thallus (Figs 4–6). These results indicate that MpRFL is essential for proper thallus and gemma cup formation. Several genes have been identified as essential for the

formation of gemma cups and gemma. The *GEMMA CUP-ASSOCIATED MYB1* (*GCAM1*) gene, encoding an R2R3-MYB-type transcription factor, is a master regulator of gemma cup formation, and *GCAM1* knockout lines are unable to form

any gemma cup and gemma (Yasui *et al.*, 2019). The *KARAPPO* gene, which encodes a ROP guanine nucleotide exchange factor (RopGEF), is also essential for gemma formation. Loss-of-function mutants of *KARAPPO* display a typical phenotype of impaired gemma formation without affecting gemma cup formation (Hiwatashi *et al.*, 2019). In addition, mutants of the *MpRKD* gene, which regulates germ cell differentiation and initiation, are defective in forming the rim of the gemma cup although gemmae still develop from the bottom of the gemma cup (Koi *et al.*, 2016; Rövekamp *et al.*, 2016). Compared with the above mutants, *Mprlf^{sc}* forms gemma on the thallus while proper gemma cup formation is inhibited. Interestingly, in the RNA-seq dataset, *Mp5g06550/KARAPPO* expression was unchanged in *Mprlf^{sc}*, while *Mp3g04030/MpRKD* and *Mp6g04830/GCAM1* expression was decreased (Table S3). It should be noted that we used plants grown for 16 d, which is sufficient time for gemma cup formation in WT plants under our growth condition. Considering these points, metabolic processes associated with MpRLF may contribute to the proper expressions of *GCAM1* and *MpRKD* during the process of gemma cup formation. In addition to the defects in vegetative development, *Mprlf^{sc}* mutants showed aberrant development in male and female reproductive organs, which is consistent with MpRLF promoter activity in reproductive organs (Figs 4, 7, 8). These results indicate that MpRLF is also essential for proper reproductive organ development. Further analyses of the spatial distribution and stage-specific expression pattern of the genes that regulate the formation of gemma cup, gemma, and reproductive organs (antheridiophore and archegoniophore) in *Mprlf^{sc}* will shed light on how MpRLF regulates the development of these organs.

The reduced thallus area observed in the overexpression lines, despite representing the opposite genetic condition to loss-of-function mutants, was an unexpected result (Fig. S5). We propose that MpRLF overexpression may alter intracellular metabolic states, disrupting the balance of cellular processes such as cell division and expansion, ultimately leading to growth defects. Gene Ontology analysis of RNA-seq data from MpRLF mutants revealed enrichment in apoplast (GO:0048046) and plasma membrane (GO:0005886), suggesting changes in membrane properties. This implies that similar alterations in membrane dynamics may occur in overexpression lines, potentially affecting signal transduction pathways that regulate cell cycle progression and expansion. Examining the expression of genes involved in cell cycle regulation (e.g. *CYC*s, *CDK*s, and *E2F*s) and cell expansion (e.g. *EXP*s, *CESA*s, and *XTH*s) in MpRLF overexpression lines could provide insights into the mechanisms underlying these growth defects. The overexpression of cytochrome *b₅*-like heme/steroid-binding domain (Cytb5-HBD) proteins has been reported to induce metabolic changes. For example, in *Malus domestica* (apple), the overexpression of MdCYB5 was accompanied by the upregulation of anthocyanin biosynthesis genes, such as MdANR, MdCHI, MdDFR, and MdUF3GT, leading to a significant increase in anthocyanin accumulation (Zhang *et al.*, 2024). Similarly, in the overexpression of the Cytb5-HBD protein MpRLF, it is highly likely that genes involved in intracellular metabolism are also altered. To gain deeper insight into

these mechanisms, future research should focus on identifying gene networks regulated by MpRLF through approaches such as RNA-seq and metabolome profiling using overexpression lines.

Phylogenetic analysis revealed that RLF orthologs form a distinct and well-supported clade, separate from other Cytb5-HBD protein families such as CB5B/D, CB5E, and CB5L (Fig. 2). This indicates that RLF represents a functionally specialized subgroup within the Cytb5-HBD family. The conservation of RLF orthologs across diverse plants, including angiosperms, gymnosperms, ferns, liverworts, and mosses, suggests that RLF function has been preserved throughout the evolution of land plants. Furthermore, the presence of RLF orthologs in streptophyte algae, such as *Chara* (charophytes), and green algae, including *Chlamydomonas* and *Volvox*, indicates that its origin predates the emergence of land plants. This broader distribution raises the possibility that RLF originally played a fundamental role in cellular or developmental processes that were later co-opted for land plant organ development. Given that our study focuses on the role of MpRLF in organ development, it would be interesting to investigate whether the ancestral function of RLF in algae was related to cell division and differentiation, stress responses, or other fundamental cellular mechanisms. Most plant species have a single RLF ortholog, implying possible functional diversification or subfunctionalization. Additionally, *A. thaliana* RLF was able to complement the *Mprlf^{sc}* mutant, suggesting that RLF-mediated regulation of organ development has been conserved across land plants. These findings suggest that heme-binding-dependent RLF-mediated organ development has remained a stable and essential feature throughout land plant evolution. On the other hand, the N-terminal disordered region of RLFs varies among different plant species (Fig. S2). While we did not analyze the disordered regions in detail in this study, we cannot rule out that the N-terminal structure also affects the function of RLF. It has been shown that a portion of the N-terminal region of rice RLF is similar to the N-terminal structure of human NADH cytochrome *b₅* oxidoreductase (Ncb5or) and adopts a helical structure when interacting with the Cytb5-HBD within the same protein (Benson *et al.*, 2024). How the helical structure contributes to the function of RLF is currently unknown, but the Cytb5-HBD-dependent change in the disordered region might be significant. While the N-terminal structure of each plant species is different, MpRLF rescued the LR formation phenotype of the *A. thaliana* *rlf* mutant, suggesting that the disordered region may not be essential for the function of RLF. These results raise further questions about the significance of the disordered region in the RLF protein family.

Our study highlights the essential role of heme-binding activity in RLF function and its necessity for proper organ development in *A. thaliana* and *M. polymorpha*. We hypothesize that heme-bound RLF acts as a regulatory mediator, enabling RLF to participate in redox reactions or protein interactions critical for developmental transitions. The evolutionary conservation of RLF function across land plants underscores the central importance of heme-binding in complex organ development. Further studies on RLF orthologs, including *A. thaliana* RLF and MpRLF, will deepen our understanding of the relationship between Cytb5-HBD

evolution and the biological roles of the RLF protein family, advancing our knowledge of conserved mechanisms in plant development.

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Competing interests

None declared.

Author contributions

KP Iwata and H Fukaki designed the study. KP Iwata performed most of the experiments. TS and TM measured the absorbance of the proteins. YS, TF, H Fukumura, and K Ishizaki supported the experiments using *M. polymorpha*. KP Iwata, TS, YS, TF, H Fukumura, YK, TM, K Ishizaki, and H Fukaki discussed the research. KP Iwata and H Fukaki wrote the manuscript, with input from all authors.

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Data availability

The data that support the findings of this study are available in the article and in Figs S1–S7 and Tables S1–S3. The RNA-seq data here generated are available on the NCBI Sequence Read Archive (SRA) (PRJNA1225401).

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- Fig. S1** Predicted structure of wild-type and mutant RLF proteins.
- Fig. S2** Alignment of the amino acid sequence of the RLF orthologs.
- Fig. S3** Expression pattern of the *Mp3g04390* (*MpRLF*) gene in Marpolbase Expression database.
- Fig. S4** Growth defects and reproductive abnormalities in *MpRLF* loss-of-function mutants.
- Fig. S5** Overexpression of *MpRLF* inhibits thallus growth.
- Fig. S6** RNA-seq analysis between wild-type and *Mprlf^{ke}* mutant.
- Fig. S7** Gene set enrichment analysis between wild-type and the *Mprlf^{ke}* mutant.
- Table S1** Primers used in this study.
- Table S2** Genes enriched in inorganic anion transport (GO:0015698) and chitin catabolic process (GO:0006032) in Fig. 11.
- Table S3** Gene expression level in the *Mprlf^{ke}* mutant.

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