

**The microRNA FEW RHIZOIDS1 controls rhizoid patterning
in the liverwort *Marchantia polymorpha***



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Thesis submitted for the degree of Doctor of Philosophy

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Abstract

MicroRNAs (miRNAs) control gene expression and consequently play regulatory roles in the development of plants and animals. The FEW RHIZOIDS 1 (MpFRH1) miRNA of the liverwort *Marchantia polymorpha* was identified as a negative regulator of rhizoid development. ROOT HAIR DEFECTIVE 6-LIKE (RSL) class I basic Helix-Loop-Helix transcription factors (TFs) positively regulate rhizoid development in bryophytes and root hair development in angiosperms, thus being conserved positive regulators of tip-growing cells with a rooting function in land plants. Here, I demonstrate that MpFRH1 directly targets the MpRSL1 mRNA, the only RSL class I TF in *M. polymorpha*. While GLABRA2 (AtGL2) negatively regulates RSL class I TF expression in the angiosperm *Arabidopsis thaliana*, the function of MpFRH1 as a negative regulator of RSL class I TFs in liverworts demonstrates that independent mechanisms evolved to repress RSL class I TF expression in different land plant lineages.

To determine how MpFRH1 represses rhizoid development I generated loss of function (LOF) mutations in the MpFRH1 gene. I showed that rhizoid initials develop larger clusters in Mpfrh1^{LOF} plants than in wildtype. Furthermore, the lack of MpFRH1 activity causes the development of supernumerary mucilage papillae cells and meristematic notches. These findings indicate that MpFRH1 suppresses the development of ectopic epidermal structures such as rhizoid cells and mucilage papillae cells as well as apical cells in meristems.

By combining functional and spatiotemporal expression data of both MpFRH1 and MpRSL1, using promoter reporter constructs, an MpFRH1 activity biosensor and *in situ* hybridizations, I suggest that MpFRH1 may act as a mobile miRNA required for lateral inhibition during epidermal patterning formation.

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Abbreviations

AGO1	-	ARGONAUTE1
APT	-	ADENINE PHOSPHORIBOSYL TRANSFERASE
ARF	-	AUXIN RESPONSIVE FACTOR
bHLH	-	basic Helix-Loop-Helix
bp	-	base pair
Cas	-	CRISPR associated protein
CLV	-	CLAVATA
CPC	-	CAPRICE
CRISPR	-	Clustered Regularly Interspaced Short Palindromic Repeats
CUL	-	CULLIN
DCL	-	DICER LIKE
DDL	-	DAWDLE
DNA	-	Deoxyribonucleic acid
EGL3	-	ENHANCER OF GLABRA3
ETC	-	ENHANCER OF CPC
GL	-	GLABRA
GRN	-	Gene Regulatory Network
HD-ZIP III	-	Class III homeodomain-leucine zipper
HEN	-	Hua Enhancer
HST	-	HASTY
HYL1	-	HYPONASTIC LEAVES1
LCR	-	LEAF CURCLING RESPONSIVENESS
MP	-	mucilage papillae
mRNA	-	messenger RNA

OC	-	organizing centre
PCR	-	Polymerase chain reaction
PHB	-	PHABULOSA
PHV	-	PHAVOLUTA
pre-miRNA	-	precursor-miRNA
pri-miRNA	-	primary-miRNA
RHD	-	ROOT HAIR DEFECTIVE
RI	-	rhizoid initial
RISC	-	RNA induced silencing complex
RNA	-	Ribonucleic acid
RSL	-	ROOTHAIR DEFECTIVE SIX-LIKE
siRNA	-	small interfering RNA
SAM	-	Shoot Apical Meristem
SAUR	-	SMALL AUXIN UP RNA
SCM	-	SCRAMBLED
SCR	-	SCARECROW
SD		Standard Deviation
SE	-	SERRATE
SHR	-	SHORTROOT
tasi-RNA	-	trans-acting small interfering RNA
TF	-	Transcription Factor
TRY	-	TRIPTYCHON
TTG	-	TRANSPARENT TESTA GLABRA
WER	-	WEREWOLF
WUS	-	WUSCHEL

Chapter 1

Introduction

The development of multicellular organisms requires the specification of cells into various types that differ in shape, size, composition and function. Cell differentiation is controlled through highly dynamic and complex Gene Regulatory Networks (GRNs). The core unit of GRNs are genes, which are encoded in the DNA. The transcription of genes into their messenger RNAs (mRNA) is activated or repressed through transcription factors (TFs) which constitute regulators in the hubs of these networks (Babu et al., 2004). Following transcription, mRNAs are translated into proteins, which trigger signalling cascades leading to cell differentiation. However, non-coding RNAs like microRNAs (miRNAs), can interfere in this process. Like protein-coding genes, miRNAs are encoded in genes harbouring their own transcription start site. Their transcripts are processed into short non-translated RNA molecules of about 21 bp. Those short RNA molecules bind to the mRNA of their target genes through complementary base pairing to either block the translational process (Chen, 2004) or cause the mRNA to be cleaved (Xie et al., 2014). Consequently, miRNAs mediate post transcriptional gene silencing to regulate the expression of TFs and vice versa (He and Hannon, 2004; Filipowicz et al., 2008).

To date, 326 miRNAs have been identified in *Arabidopsis thaliana* and uploaded to miRBase (<http://www.mirbase.org/>), a database that acts as an archive of microRNA sequences and annotations (Kozomara and Griffiths-Jones, 2011). Since miRNAs bind to their target genes through complementary base pairing, computational sequence analysis allows the identification of putative target genes, which subsequently require experimental validation. Several miRNAs have been validated and characterised *in planta*, revealing their elusive roles in plant development, but also in signal transduction, stress tolerance, biosynthesis of secondary metabolites and many more (Benkovics and Timmermans, 2014; Liu et al., 2017; Samad et al., 2017). In section 1.1, I will describe prominent examples in

which the interaction of miRNAs and their target genes are studied in great detail, highlighting their versatile mechanisms and their importance in plant development.

In early diverging lineages of land plants, conserved miRNA-target gene interactions have been identified (Lin and Bowman, 2018). However, the majority of miRNAs identified are not conserved throughout land plants, suggesting that some miRNAs evolved very early during land plant evolution and remained functional while most miRNAs evolved independently in specific land plant lineages (Lin and Bowman, 2018). These findings indicate the role of miRNAs as dynamic and highly adaptive evolutionary drivers (Rajagopalan et al., 2006, Fahlgren et al., 2007, Axtell and Bowman, 2008, Lu et al., 2008, Lyu et al., 2014). I will summarize current findings of conserved and non-conserved miRNAs in section 1.2.

During my DPhil I investigated the function of a novel liverwort specific miRNA and how it controls the development of the liverwort *Marchantia polymorpha*. In particular, I studied how it affects the formation of specialised structures developing from epidermal cells, e.g., rhizoid cells and mucilage papillae cells. In section 1.3 I will describe the development of *M. polymorpha*, starting with its life cycle and focussing on the development those specialised epidermal structures. Rhizoids and root hairs (specialised root epidermal cells growing on sporophytes of vascular plants) are analogous cells and their developments underlie a partially conserved molecular mechanism. Section 1.4 provides a report on the current knowledge about the molecular mechanism controlling root hair development in *A. thaliana*.

1.1 miRNAs and TFs are major components that control cell differentiation in plants

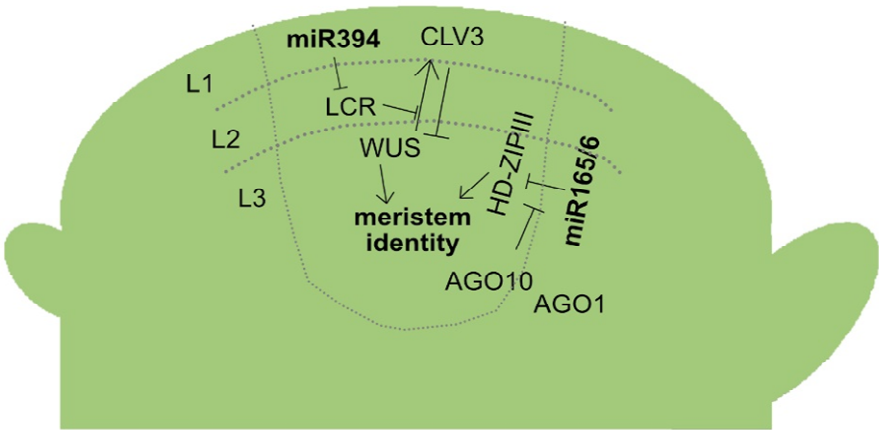
MiRNAs modulate the activity of gene regulatory networks that control developmental processes such as the patterning of the embryo, meristem, leaf, root and flower. Important examples of miRNA activity during plant development are summarized in the following sections to highlight their precise modes of action to control the spatiotemporal expression of their target genes.

miR394 and miR165/6 restrict meristematic cells to the organizing centre of the SAM

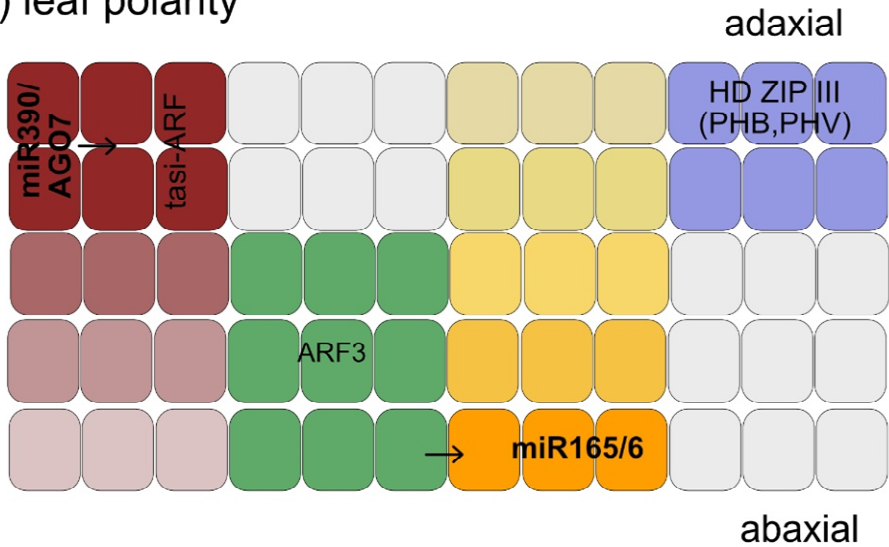
In *Arabidopsis*, the shoot apical meristem (SAM) is made of a pool of pluripotent stem cells within the first three layers at the tip of the shoot (L1-L3, central zone, Satina et al., 1940). The L1, or protoderm, and L2 give rise to epidermal and subepidermal cells through anticlinal cell divisions, respectively. A small group of cells within the L3 constitute the organizing centre (OC) which are undifferentiated stem cells that give rise to the inner corpus tissue (Haecker and Laux, 2001, Boscá et al., 2011). The maintenance of the OC is controlled by the expression of the homeobox TF WUSCHEL (WUS, Mayer et al., 1998). WUS moves through plasmodesmata (Daum et al., 2014) dorsally into adjacent cells of the central zone to activate its own negative regulator, the peptide CLAVATA3 (CLV3). CLV3 in turn moves inwards again to suppress WUS activity via a receptor kinase signalling cascade (Jun et al., 2008) and hence builds a negative feedback loop. The restriction of WUS to the OC is enforced through miR394 activity, which is produced in the L1 layer. From there it diffuses basally towards the OC and suppresses LEAF CURLING RESPONSIVENESS (LCR), a F-box protein that represses WUS mediated CLV3

activation. Consequently, WUS expression is suppressed in cells outside the OC to restricts the OC to a small group of cells only (Knauer et al., 2013, Figure 1.1a).

A) meristem maintenance



B) leaf polarity



C) radial root patterning

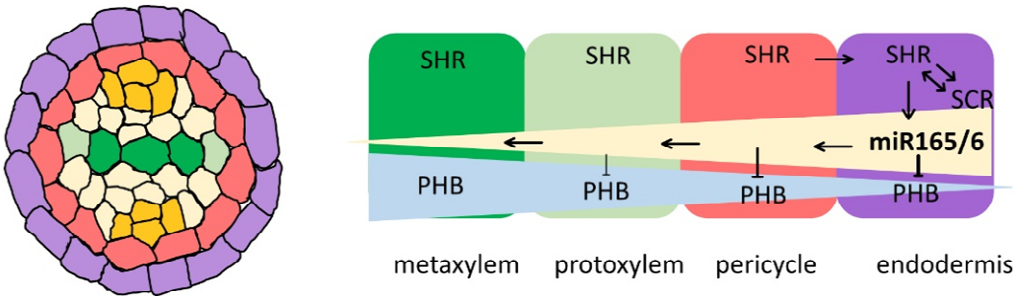


Figure 1. 1 MiRNA activity during plant development

A) SAM of *A.thaliana*: WUS and HD-ZIP III activity maintains stem cell and meristem identity in the organisation centre (OC, within L3). WUS moves toward L1 to positively regulate CLAVATA 3, which in turn represses WUS and restricts WUS activity to the OC. The restriction to the OC is reinforced through miR394 activity, which is produced in the L1 from where it moves basally towards the OC (within L3). It targets *LCR* mRNA, which suppresses WUS activity outside the OC. MiR165/6 suppresses HD-ZP III activity outside the SAM (through mRNA cleavage mediated by AGO1). When miR165/6 diffuses into the SAM, miR165/6 gets sequestered by AGO10 and HD-ZIP III expression is not repressed by miR165/6. Dotted lines mark L1, L2, L3 and the border between SAM and the periphery.

B) Schematic overview of leaf primordium: miR390 together with AGO7 generates tasi-ARF in the two adaxial cell layers of a leaf primordium. Tasi-ARF, which targets ARF3 mRNA, diffuses abaxially to generate a concentration gradient (gradient indicated from dark to light red). This gradient is translated into a sharp ARF expressing (green) and ARF non-expressing (grey) cell boundary. ARF activates the production of miR165/6 in the most abaxial cell layer which diffuses to the adaxial cells, generating a concentration gradient in the opposite way to tasi-ARF (gradient indicated from dark orange to light orange). MiR165/6 targets HD-ZIP III mRNA and its activity generated a sharp on-off boundary of HD-ZIP III expressing (blue) and non-expressing cells (grey).

C) Cross section of developing Arabidopsis root and individual cell types along the xylem axis: SHR is produced in the stele and moves to the endodermis, where together with SCR it activates the production of miR165/6, which targets *PHB* mRNA. The miRNA moves backwards to the stele generating a concentration gradient (indicated in beige). This gradient gets translated into an inverse gradient of PHB activity (indicated in blue). Cells with very low PHB activity differentiate into endodermis cells, of medium concentration into pericycle cells and of low concentration into stele cells (proto- and metaxylem).

MiR165 and miR166 (miR165/6) belong to another important class of miRNAs involved in restricting the meristem to the central zone. MiR165/6 are produced in the peripheral zone outside the central zone to suppress the accumulation of Class III homeodomain-leucine zipper (HD-ZIP III) TFs and thus ectopic meristem initiation (since HD ZIP IIIs trigger meristem identity). MiRNA activity is guided through ARGONAUTE1 (AGO1), which is part of the RNA induced silencing complex (RISC) that binds single stranded

miRNAs and mediates cleavage of target mRNA. AGO1 is expressed throughout the shoot apical meristem and ensures miR165/6 mediated suppression of HD ZIP III mRNA. AGO10, an AGO1 homolog, is only expressed in the central zone of the SAM (Newman et al., 2002). By contrast, binding of miR165/6 to AGO10 does not result in degradation of HD-ZIP III mRNA. Therefore, a function of AGO10 as a decoy protein, has been proposed (Zhu et al., 2011; Zhou et al., 2015). AGO10 sequesters miR165/6 in the central zone and hence blocks their activity. Therefore, miRNA165/6 that diffuse from the peripheral zone into the central zone is sequestered by AGO10 and no miR165/6 mediated cleavage of HD-ZIP III mRNA takes place in the meristematic region (Zhou et al., 2015). Thus, HD-ZIP III expression is restricted to the central zone to promote meristem identity (Figure 1.1a).

MiR165/6 and miR390 act antagonistically to restrict HD-ZIP III and ARF3 to cause sharp ON-OFF boundaries during leaf polarity establishment

After leaving the central zone to the periphery, some cells start differentiating into leaf primordia, which will develop into leaves. During leaf development adaxial-abaxial polarity is established. The abaxial domain is defined through the activity of trans-acting small interfering RNAs (tasi-RNAs) targeting AUXIN RESPONSIVE FACTOR 3 (ARF3) and ARF4 (Allen et al., 2005; Williams et al., 2005). tasiR-ARF biogenesis is restricted to the two dorsal- most cell layers (e.g. abaxial). Here, miR390-AGO7 complex cleaves the TAS3 transcript, which subsequently allows the generation of double stranded RNA. DCL4 processes the dsRNA into 21nt long phased small interfering RNAs (siRNAs), e.g. tasiR-ARFs. Mature tasi-ARF moves to the ventral side and generates a concentration gradient. This gradient is interpreted into regions of high and low tasiR-ARF activity. This causes ARF3 expression to be completely blocked on the dorsal side and a uniform level of ARF3

on the ventral side, forming a sharp boundary of ARF3 expressing and non-expressing cells (Skopelitis et al., 2017, Figure 1.1b).

ARF3 activity is auxin-dependent and promotes transcription of the miR165/166 genes (Nogueira et al., 2007) in the ventral epidermis. miR165/6 in turn, move towards the dorsal side and cause cleavage of their HD ZIP III target genes *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*), which are transcribed uniformly throughout the leaf. Similar to tasi-ARF3 this gradient generates a sharp on-off boundary of PHB/PHV expressing and non-expressing cells (Benkovics and Timmermans, 2014; Skopelitis et al., 2017) in the opposite manner. Consequently, PHB and PHV expression defines the adaxial domain (Figure 1.1b).

miR165/6 generate a gradient to control radial patterning in root development

While miR165/6 generate a sharp on-off boundary of their HD-ZIP III targets during leaf development, their interaction generates a gradient of HD-ZIP III expression during root development in *A. thaliana*. The root consists of a central vascular stele (phloem, xylem cells) surrounded by concentric rings of pericycle, endodermis, cortex, and epidermis. SHORTROOT (SHR) is expressed in the vascular stele and moves into the endodermis, where it positively regulates the transcription factor SCARECROW (SCR) (Nakajima et al., 2001). SHR and SCR interact to form a positive feedback loop that further activates SCR transcription (Cui et al., 2007; Cruz-Ramírez et al., 2012). In the endodermis, SHR activates transcription of *miR165/6* (Carlsbecker et al., 2010). Mature miR165/6 in turn move back to the stele and generate a concentration gradient and reduce *PHABULOSA* (*PHB*) mRNA abundance in an inverse HD-ZIP III gradient; cells at different positions along this gradient develop pericycle, protoxylem and metaxylem identity (Carlsbecker et

al., 2010). This suggests that a dose-dependent readout of a small RNA gradient drives the acquisition of discrete cell fates (Figure 1.1c).

Genomic and transcriptomic analysis among diverse land plant lineages revealed that core components of the miRNA biogenesis pathways are conserved among land plants. Only a small number of mature miRNAs are shared by most land plants. Most miRNAs are family- or species- specific, suggesting that most miRNAs originated relatively recently in land plants (Cuperus et al., 2011; Lin et al., 2016; Tsuzuki et al., 2016; You et al., 2017).

1.2 Conserved and non-conserved miRNAs among land plants

MiRNAs are pervasive regulators in both plant and animals development (Axtell et al., 2011). Many aspects of their biogenesis, function and evolution differ and the lack of sequence homology between miRNA families in plants and animals support the hypothesis that miRNAs have evolved independently between plants and animals (Millar and Waterhouse, 2005; Shabalina and Koonin, 2008; Moran et al., 2017). Most of our knowledge about miRNA biogenesis and function in plants comes from studies in the angiosperms *A. thaliana* and *Oryza sativa*, which is summarised in the following paragraphs.

MiRNAs are transcribed by RNA polymerase II, producing the primary miRNA (pri-miRNA), which folds back on themselves through imperfect, self-complementary sequences to form a stem loop double stranded RNA. It is presumed that the RNA-binding protein DAWDLE (DDL) stabilizes folded pri-miRNAs for further processing (Yu et al., 2008). DICER LIKE1 (DCL1) endoribonucleases orchestrate the processing events within the nucleus (Papp et al., 2003). Involvement of the C₂H₂-zinc finger protein SERRATE (SE), the double-stranded RNA-binding protein HYPONASTIC LEAVES1 (HYL1) (Grigg et al., 2005, Kurihara et al., 2006, Dong et al., 2008) and other RNA-binding

proteins ensures accurate and efficient cleavage. This protein complex (DCL – SE- HYL1) binds to the hairpin structure and DCL1 first catalyses cleavage of the foldback loop- distal side, generating the precursor miRNA (pre-miRNA) followed by a second cut at the loop-proximal side, resulting in an ~21-nucleotide mature miRNA/miRNA passenger strand (miRNA*) duplex with 3' overhangs of two nucleotides. Mature miRNA duplexes are stabilized by the S-adenosylmethionine-dependent methyltransferase Hua Enhancer 1 (HEN1), which methylates the overhanging 3'OH groups (Park et al., 2002, Yang et al., 2006). Presumably the miRNA/miRNA* duplex then is exported from the nucleus to the cytoplasm. Currently HASTY (HST), an exportin 5 ortholog (Bollman, 2003), and other unknown factors are believed to be involved in the nuclear export process (Park et al., 2005). MiRNA* is degraded while the mature miRNA is incorporated into the RNA-induced silencing complex (RISC), which guides the mature miRNA to its target mRNA to form a miRNA-mRNA interaction. This causes either irreversible cleavage of the target mRNA by the activity of AGO proteins or reversible blockage of the translational machinery at that mRNA (Voinnet, 2009, see figure 1.2). While plant miRNAs primarily regulate their target genes by mediating mRNA cleavage at single sites in the coding region, animal miRNAs primarily cause post transcriptional silencing by mediating translational repression through multiple miRNA binding sites that are located within the 3' UTR of the mRNA (Millar and Waterhouse, 2005). The mode of mechanism correlates with the degree of complementarity between miRNA and target mRNA. Most animal miRNAs have lower complementarity to their target mRNA (and mediate translational inhibition), while most plant miRNAs have high complementary to their target mRNAs (and mediate mRNA cleavage) (Millar and Waterhouse, 2005).

Comparative genomic studies revealed that core components of the miRNA biogenesis pathways, including AGO and DCL proteins, are conserved in the earliest

diverging lineages of land plants, e.g. *M. polymorpha* and *Physcomitrella patens* (Lin et al., 2016, Tsuzuki et al., 2016, Axtell et al., 2007). The *M. polymorpha* genome harbours one DCL1, one DCL3 and one DCL4 ortholog as well as one AGO1-like and two AGO4-like proteins. *P. patens* has four DCL proteins (PpDCL1a/b, PpDCL3 and PpDCL4) and six AGO proteins (PpAGO1a/b/c and PpAGO4a/b/c). No HEN1 orthologue could be identified in *M. polymorpha* but it was shown that miRNAs are methylated nevertheless. This indicates that the miRNA biogenesis pathway has been evolved early in land plant evolution and the core machinery is conserved.

Two independent studies investigated the presence of miRNAs among land plants. Lin et al. (2016) characterised miRNAs and their mRNA targets in *M. polymorpha* using computational, transcriptomic, small RNA and degradome analyses. The identified miRNAs were then compared to miRNAs from *P. patens*, *Selaginella moellendorffii*, *Picea abies*, *Pinus taeda*, *A. thaliana* and *O. sativa* (registered in miRbase or published elsewhere). Tsuzuki et al. (2016) identified miRNAs expressed in thalli, antheridiophores and archegoniophores in *M. polymorpha* and compared those ones with miRNAs from *P. patens* and *A. thaliana* registered in miRbase.

Taken together both studies, there are 7 conserved miRNA families, namely miR166, miR390, miR171, miR319 miR160, miR408 and miR529 among land plants with both ancestral and novel targets. For example, miR160 targets ARF mRNAs in *A. thaliana* and ARF3 mRNA in *M. polymorpha*, and miR166 targets Class III HD-ZIP mRNA in *A. thaliana* and *M. polymorpha*. A conserved miRNA-mRNA interaction among land plants suggests that particular developmental processes might have been controlled through a conserved miRNA-target gene dependent mechanism since the first land plants evolved.

Two miRNAs were identified as bryophyte specific, hence present in *P. patens* and *M. polymorpha* but absent in *A. thaliana* (e.g. miR536 and miR1030). But the majority of

miRNAs (e.g. 115 miRNAs) that were identified in their studies were only present in *P. patens*, or *M. polymorpha*, indicating that miRNAs and miRNA-mRNA interactions can be unique to individual phylogenetic groups.

The miRNA MpFRH1 is one example of a liverwort specific miRNA that has been identified in a T-DNA mutagenesis screen of rhizoid defective mutants in *M. polymorpha* (Honkanen et al., 2018). In the transcriptomic analysis mentioned above (Tsuzuki et al., 2016) MpFRH1 corresponds to the miRNA Mpo-MIR11861 that is expressed at low levels in 4-week-old thalli and the archegonial head. Overexpression of this miRNA suppresses the formation of rhizoid cells suggesting that MpFRH1 is a negative regulator of rhizoid cell development (Honkanen et al., 2018). This DPhil thesis focusses on the functional characterization of this miRNA during the development of *M. polymorpha* and in particular during the differentiation of specialised epidermal structures like rhizoid cells and mucilage papilla cells. In the last section of the introduction, I will describe the development of *M. polymorpha*, starting with its life cycle and focussing on the development of specialised epidermal structures like rhizoid cells and mucilage papilla cells.

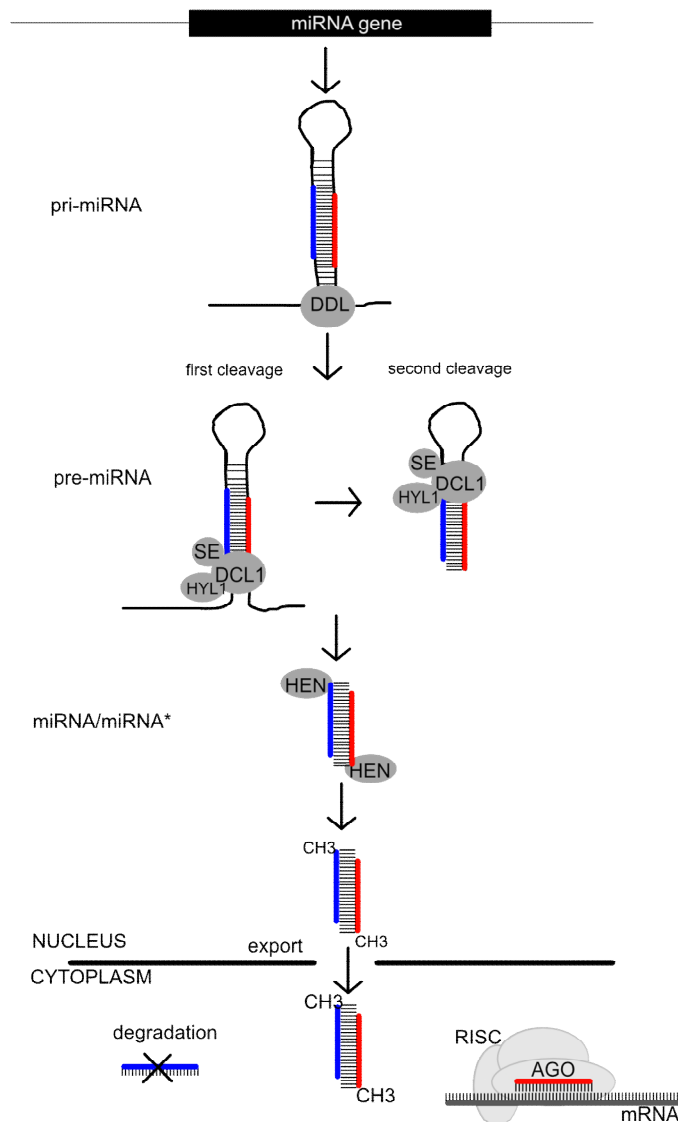


Figure 1. 2 MiRNA processing pathway in plants

MIR genes are transcribed into primary miRNA (pri-miRNA), which folds into a hairpin structure due to complementary sequences of miRNA (red) and miRNA* (blue), which is stabilized by DDL. DCL1, with the involvement of SE and HYL1 processes the pri-miRNA through cleavage at the base of the hairpin. A second cleaving event by DCL1 at the loop of the hairpin generates the miRNA/miRNA* duplex. HEN methylates the 3'OH ends. The duplex is transported into the cytoplasm where miRNA* gets degraded and the miRNA loaded into the RISC. The loaded RISC is guided to the target mRNA through base pair complementarity, where AGO mediates cleavage of the target mRNA. Alternatively, the RISC blocks the translational machinery. Both mechanisms mediate post transcriptional silencing.

1.3 Development of *M. polymorpha*

M. polymorpha is a member of the liverwort lineage, which is one of the three earliest diverging lineages of land plants (besides mosses and hornworts) that emerged some time before 450Mya (Rubinstein et al., 2010; Morris et al., 2018). Consequently, it holds a key phylogenetic position for comparative studies with other land plants allowing us to address several aspects regarding the evolution of gene networks and its functional conservation. In *M. polymorpha*, most TF families consist of very few members compared to other land plants (Catarino et al., 2016; Bowman et al., 2017). These low numbers are advantageous for studying gene function and therefore developmental processes and their GRNs in this species (Pires and Dolan, 2010, Berger et al., 2016, Proust et al., 2016, Breuninger et al., 2016, Jones and Dolan, 2017, Bowman et al., 2017). *M. polymorpha* is easy to transform and to genetically manipulate (Ishizaki et al., 2008). Furthermore, in liverworts the haploid generation of the life cycle is dominant. Therefore, in a mutant screen the defective phenotype caused by the loss-of-function or gain-of-function of a gene can be identified within the first generation without the establishment of homozygous lines opposite to plants in which the diploid generation is dominant, such as in *A. thaliana*. *M. polymorpha* offers many benefits as a model system to study molecular and developmental processes including its short life cycle, ease of propagation (through asexual vegetative propagules called gemmae) and the ability to make controlled crossings, because it is a dioecious species (with male and female individuals). Molecular techniques are available, including homologous recombination, artificial miRNA and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR associated protein 9 [Cas9]) to introduce target directed mutations (Ishizaki et al., 2013; Sugano et al., 2014; Flores-Sandoval et al., 2015a; Shimamura, 2015).

The dominant life stage in *M. polymorpha* is the haploid gametophyte

Like all land plants, *M. polymorpha* exhibits alternation of generations which is illustrated in Figure 1. 3. In *M. polymorpha* the dominant generation consist of the multicellular haploid gametophyte, also called the thallus. The development of the thallus begins with a unicellular spore that germinates and forms an initial short filamentous ‘protonema’ with rhizoids. Through irregular cell divisions of protonema cells a spherical protonema develops. A lenticular-shaped apical cell is formed and undergoes several oblique divisions with two cutting faces to produce a unistratose thallus-like region. This apical cell is converted to a cuneate-shaped apical cell by addition of dorsal and ventral division planes and becomes the growing point located in the apical notch of the thallus. Here new thallus tissue develops, and through periodic bifurcation new axes of growth rise resulting in the characteristic growing pattern of the *M. polymorpha* thallus (Shimamura, 2015; Solly et al., 2017).

Since *M. polymorpha* is a dioecious plant (Bischler and Boisselier-Dubayle, 1993), female gametophytes give rise to archegoniophores while male gametophytes give rise to antheridiophores (Shimamura, 2015) on the dorsal side of the thallus. These structures produce female and male gametangia, called archegonia and antheridia respectively. A single egg is produced in the venter of each archegonium that is fertilized by a motile sperm produced from the antheridia. The fertilised egg cell develops into a very short living diploid sporophyte which is dependent on the gametophyte. Within the sporophyte, meiotic divisions of spore mother cells (or sporocytes) lead to the production of haploid spores, which subsequently germinate to form a new male or female gametophyte (O’Hanlon, 1926).

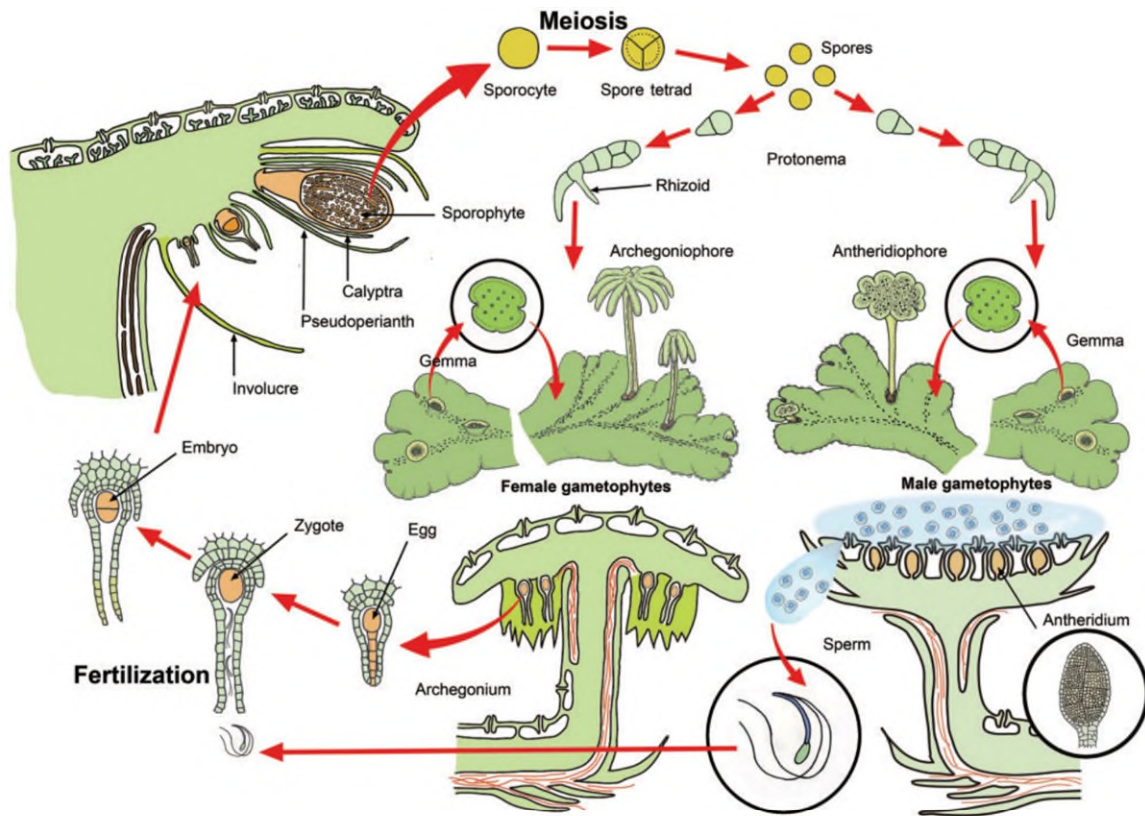


Figure 1. 3 Life Cycle of *Marchantia polymorpha* (Shimamura, 2015)

The gametophytes constitute the dominant generation while the sporophyte is a small and simple structure, whose development depends on the female gametophyte. The male gametophyte forms antheridiophores (harbouring the antheridia, producing antherozoids (sperm)). The female gametophyte forms archegoniophores (harbouring archegonia, each of which contains a single egg cell). After fertilization the sporophyte forms and remains attached to the archegoniophore. Spores are formed in the sporophyte through meiosis. They are released and develop into new gametophytes.

M. polymorpha can also reproduce asexually through the production of vegetative multicellular propagules called gemmae. They are produced in gemma cups (Bowman, 2016), which typically appear once in each bifurcation on the dorsal side of the mature thallus (see Figure 1.3). Gemmae are continuously produced from the bottom of the cup from individual epidermal cells. Gemma initials at the bottom of the gemmae cup elongate and divide transversely to give rise to a stalk cell and a gemma cell. While the stalk cell does not divide further, the gemma cell divides transversely to form filamentous tiers (Figure 1.4A). Further divisions (vertically) form a flattened early gemma. Two potential apical cells appear on opposite sides of the gemma (Figure 1.4B). Through active cell divisions of the derivatives of the apical cells, the characteristic notch forms (Figure 1.4C). The mature gemma is usually approximately 300 – 500µm in diameter, with two apical notches harbouring the apical cell which are covered by mucilage hair. Specialised cells such as oil cells and rhizoid initials are differentiated on the epidermal surface.

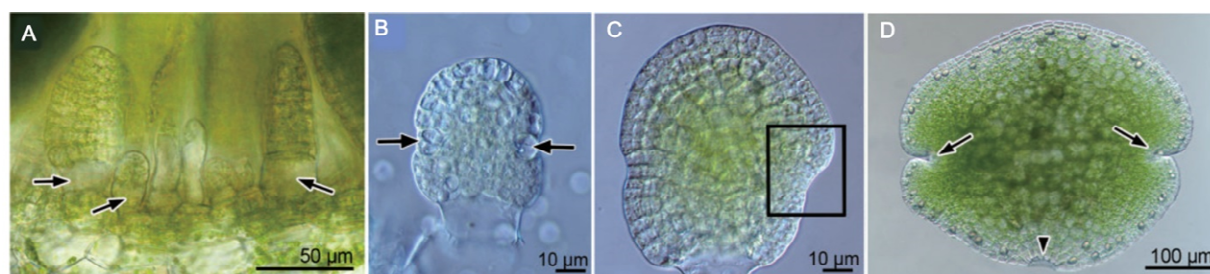


Figure 1. 4 Gemma development

A) Vertical section of the bottom of a gemma cup showing very early stages of developing gemmae. Arrows indicate the single-celled stalk. Cell divisions of the gemma cell gives rise to filamentous tiers. B) Disk-like early gemma with two potential apical cells forming (arrows). C) Formation of apical notches (squared area) through the activity of apical cells. D) mature gemma with two apical notches (arrows) and the stalk attachment cells (arrowhead)

Apical and subapical cells that are protected by mucilage hair cells are the source of cells for the thallus

Each apical notch of a mature gemma harbours one apical cell similar to the apical notch of a mature thallus (Halbsohn, 1936, Shimamura, 2015). Here, a single cuneate apical cell with four cutting faces serves as a generative centre. It gives rise to a dorsal, a ventral and two lateral cells. The dorsal cell constitutes the initial cell of the dorsal merophyte, giving rise to dorsal epidermis of the midrib region, and occasionally gemmae and gemma cups (Shimamura, 2015). The ventral cell constitutes the initial cells of the ventral merophyte, from which no specialised tissue is differentiated (ventral epidermis only). The lateral cells become subapical cells, from which all types of tissue constituting a thallus are differentiated by regular cell divisions. The subapical cells are similar to the apical cell in shape, dividing in the same way than the apical cell, producing derivative cells in the dorsal, ventral and lateral direction. The ventral derivative further divides to form the protodermal and subprotodermal cells. The protodermal cell protrudes outward and undergoes a transverse division. Part of the initial protuberance becomes a mucilage papilla and the other part forms a ventral scale. Those structures, ventral scales and mucilage papillae, form a protective shield above the apical cell (Renzaglia et al., 2000). The derivatives on the dorsal side also divide and protodermal and subprotodermal cells layer are produced. Later in development air chambers form below the dorsal epidermis (Shimamura, 2015) and rhizoids in the ventral epidermal (Cao et al., 2014).

Rhizoids are specialised epidermal cells

Rhizoids are unicellular filamentous tip growing cells developing from individual ventral epidermal cells. There are two different types of rhizoids: smooth rhizoids and pegged rhizoids (Cao et al., 2014). While smooth rhizoids are growing down to the substrate to absorb water and nutrients and anchor the plant body to the soil, pegged rhizoids usually grow in parallel to the ventral surface. They grow as bundles and are covered by ventral scales. Those bundles function as external water-conducting systems by capillarity. On a mature gemma, rhizoid initials (RIs) - enlarged chlorophyll-less cells - are formed on both epidermal sides since no dorsoventrality has been established yet (Mirbel, 1835, Bowman, 2016). Once the gemma is in contact with the substrate, smooth rhizoid cells begin to develop from both sides through tip growth of the RIs (Taren, 1958; Honkanen et al., 2016). Once dorsoventrality is established rhizoid cells are only forming on the ventral epidermis (Cao et al., 2014; Bowman, 2016).

RSL class I genes control rhizoid development in bryophytes and root hair formation in angiosperms

Morphologically and functionally rhizoids of bryophytes are similar to root hair cells in tracheophytes. While rhizoids grow out from individual epidermal cells on the haploid gametophyte, root hairs develop from root epidermal cells on the diploid sporophyte. Both rhizoid and root hair development are controlled by the activity of ROOTHAIR DEFECTIVE SIX-LIKE (RSL) class I basic helix-loop-helix (bHLH) TF. The *M. polymorpha* genome encodes one member of the RSL class I, MpRSL1. *M. polymorpha* plants lacking MpRSL1 function do not develop rhizoids. Overexpression of MpRSL1 causes the development of ectopic rhizoids on ventral and dorsal sides (Proust

et al., 2016). In *A. thaliana* there are two RSL class I bHLH TFs, ROOTHAIR DEFECTIVE 6 (AtRHD6, At1G66470) and RHD6 – LIKE 1 (RSL1; At5G37800). *Atrhd6* mutants develop only few root hairs while root hair formation in *Atrsl1* single mutants is not affected. The *Atrhd6 Atrsl1* double mutant does not develop root hairs (Menand et al., 2007), indicating that RSL class I bHLH TFs are required for root hair formation and AtRHD6 and AtRSL1 have partially redundant functions during root hair formation. Root hair development can be restored in *Atrhd6 Atrsl1* double mutants by expressing *MpRSL1*, showing conservation of the molecular function of RSL class I proteins in the last common ancestor of land plants before the divergence of bryophytes and vasculature plants (Proust et al., 2016). The observation that RSL class I genes control rhizoid as well as root hair formation also suggests that common genetic components control the development of specialised epidermal cells with rooting function in the haploid gametophyte as well as in the diploid sporophyte. This implies that during the transition from gametophyte to sporophyte dominance a similar genetic mechanism became recruited to execute similar functions. This idea is supported by similar findings regarding polar auxin transport and water conducting cells (Kenrick, 2017).

1.4 Genetic basis of root hair formation in *A. thaliana*

In previous studies, the gene regulatory network controlling root hair formation in *A. thaliana* has been investigated to great detail (reviewed in Datta et al., 2011) and is briefly summarized in the following.

Whether a root epidermal cell differentiates into a root hair cell (H cell) or a non-root hair cell (N cell) is controlled by the activity of the class IV HD- ZIP TF GLABRA2 (AtGL2). When AtGL2 is expressed it suppresses the differentiation into H cells, while root epidermal cells not expressing AtGL2 differentiate into H cells (Masucci

et al., 1996). The expression of *GL2* is promoted by a complex consisting of *TRANSPARENT TESTA GLABRA1* (*TTG1*), *GLABRA3* (*GL3*), *ENHANCER OF GLABRA3* (*EGL3*) and *WEREWOLF* (*WER*) in N cells (Galway et al., 1994; Lee and Schiefelbein, 1999; Bernhardt et al., 2003). *TTG1* encodes a WD40 protein, *GL3/EGL3* encode bHLH TFs and *WER* encodes a R2R3 Myb TF. *WER* directly binds to the promoter of *GL2* to activate its transcription in N cells and thus suppress root hair differentiation (Lee and Schiefelbein, 1999; Koshino-Kimura et al., 2005). The R3 Myb proteins *CAPRICE* (*CPC*), *TRIPTYCHON* (*TRY*) and *ENHANCER OF CPC* (*ETC*) act redundantly and compete with the R2R3 Myb protein for interaction with the bHLH proteins *GL3* and *EGL3* (Kirik et al., 2004). The expression of *WER* is suppressed in H cells through the activity of *SCRAMBLED* (*SCM*), through underlying positional cues (Kwak et al., 2005). Hence, *CPC* which is present in all epidermal cells, can bind to *GL3/EGL3* in H cells. *CPC* acts also non-cell autonomously and moves from N into H cells which reinforces the interaction of the *GL3/EGL3-TTG* complex with *CPC* in H cells and with *WER* in N cells (Wada, 2002). When *CPC* is bound the complex does not promote *GL2* expression, and root hair initiation occurs (Ishida et al., 2008). *GL2* directly suppresses the transcription of the RSL class I genes *AtRHD6* and *AtRSL1* (Lin et al., 2015).

RSL class I proteins activate the expression of RSL class II genes (*RSL2* to *5*). *AtRHD6* and *AtRSL1* are expressed during root hair initiation and positively regulate the transcription of *AtRSL2* and *AtRSL4*, which are expressed specifically during root hair growth (Yi et al., 2010). *AtRSL4* is necessary and sufficient for root hair elongation and is therefore likely to be a master regulator of root hair cell size (Datta et al., 2015). *AtRSL3* and *AtRSL5* control root hair tip growth and are expressed during root hair elongation (Yi et al., 2010, Pires et al., 2013).

The gene regulatory network comprised by RSL class I proteins in early diverged land plants is unknown. The *M. polymorpha* genome encodes one member of class IV HD-ZIP TF (Zalewski et al., 2013; Catarino et al., 2016), but nothing is known about its function during liverwort development. In Arabidopsis, the class IV HD-ZIP TF AtGL2 represses RSL class I gene expression by directly binding L1 box sequences on the promoters of RSL class I genes (Lin et al., 2015). The genomic sequence 3.5 kb upstream of MpRSL1 however does not contain any L1-box sequences. Furthermore, GL2 homologs in many other vascular plants are not expressed in roots (Huang et al., 2017). Therefore the role of GL2 as negative regulator of RSL class I genes is not likely to be widely conserved among land plants. Together these evidences suggest that different mechanisms are likely to have evolved in bryophytes to control the expression of RSL class I TFs. A promising novel regulator of MpRSL1 was identified in a T-DNA mutagenesis screen for rhizoid defective mutants in the Dolan lab. Current evidence suggests that MpFRH1 is a miRNA that represses the expression of *MpRSL1*.

This thesis aims to validate MpFRH1 as a miRNA controlling MpRSL1 expression (chapter 1). In chapter 2 I investigate how MpFRH1 functions during plant development, in particular during rhizoid formation. My findings indicate that MpFRH1 acts non-cell autonomously, generating a lateral inhibitory field, to control the spatial distribution of rhizoid cells. This hypothesis is further tested in chapter 3, by exploring the spatial expression pattern of MpFRH1 and MpRSL1. My findings lead to the hypothesis that MpRSL1 activates MpFRH1 in emerging rhizoid cells, which suppresses the development of further rhizoids in neighbouring epidermal cells, e.g. establishing an inhibitory field around emerging rhizoid cells. This constitutes the foundation of first insights into developmental processes regulated by miRNAs in one of the earliest divergent lineages of land plants, revealing a potentially new mechanism of tissue patterning.

Chapter 2

The miRNA MpFRH1 targets Mp*RSL1* to control rhizoid and gemmae formation in *Marchantia polymorpha*

2.1 Abstract

The bHLH TF encoded by *RSL* class I genes positively regulate the development of filamentous rooting cells – root hairs and rhizoids – in land plants. In the liverwort *M. polymorpha* MpRSL1 regulates not only the outgrowth of rhizoid cells, it also regulates the formation of other structures originating from epidermal cells, e.g. mucilage papilla cells and gemmae. MpFRH1 was identified as a liverwort specific miRNA that is a negative regulator of those structures. Mp*RSL1* is among the 7 predicted target mRNAs of MpFRH1, which leads to the hypothesis that MpFRH1 is a negative regulator of MpRSL1 to control the development of epidermal structures.

This chapter describes the investigation of all 7 predicted target genes using semi-quantitative PCR, followed, if applicable, by quantitative real time PCR and 5'RLM-RACE. Mp*RSL1* could be verified as the only direct target of MpFRH1. Hence the rhizoidless phenotype of Mp*FRH1*^{GOF} is likely due to MpFRH1 directed downregulation of Mp*RSL1*. This hypothesis was confirmed through the restoration of rhizoid and gemmae development in Mp*FRH1*^{GOF} lines expressing an Mp*RSL1* transgene which is resistant to MpFRH1 binding.

In Arabidopsis the homeodomain protein GLABRA 2 has been identified as a negative regulator of RSL class1 gene transcription while no miRNA has been described to target *RSL* class 1 mRNA. These findings indicate while *RSL* class I genes are ancient and conserved, independent negative regulators have originated during land plant evolution in distant related lineages.

2.2 Introduction

In a T-DNA mutagenesis screen of rhizoid defective mutants in the liverwort *M. polymorpha*, ROOT HAIR DEFECTIVE SIX-LIKE (RSL) class I bHLH TF was identified as a positive regulator of the development of unicellular and multicellular structures that develop from individual epidermal cells, e.g. rhizoids (ventral side of the thallus), slime papillae (at the bottom of gemma cups), mucilage papillae (around meristems), or gemmae (growing from bottom of gemma cup) (Proust et al., 2016, Shimamura, 2015). *Mprsl1*^{LOF} lines usually lack the development of those epidermal structures. Hence thalli remain rhizoidless and no gemmae develop in gemma cups. Furthermore no slime or mucilage papillae develop in *Mprsl1*^{LOF} mutants. In contrast, *MpRSL1*^{GOF} lines develop rhizoids from almost any epidermal cell, while the development of slime papillae, mucilage papillae or gemmae is not defected in comparison to wildtype plants. Hence, MpRSL1 is required for the development of specialised epidermal structures and is sufficient to promote the development of ectopic rhizoid cells. In contrast, the miRNA FEW RHIZOIDS1 (MpFRH1) is a negative regulator of the development of these epidermal structures. *MpFRH1*^{GOF} lines develop very few rhizoids, mucilage papillae and gemmae (the development of slime papillae was not investigated, [Honkanen et al., 2018]). Since those plants show a very similar defective phenotype compared to *Mprsl1*^{LOF} lines, they are indicating that MpRSL1 and MpFRH1 both control the development of specialised epidermal structures and might act in the same pathway. I hypothesise that MpFRH1 is a negative regulator of MpRSL1 expression to control the development of epidermal structures.

Plant miRNAs are short ~21bp RNA molecules that bind to mRNAs harbouring a nearly complementary RNA sequence (Rhoades et al., 2002). The binding of miRNA to mRNA can cause post transcriptional gene silencing due to either promoting the cleavage of the mRNA through AGO proteins or inhibiting the translational machinery and hence blocking translation (D'Ario, Griffiths-Jones, & Kim, 2017, Chen, 2004). Target sites of miRNA in plants show high complementary with usually three mismatches (Rhoades et al., 2002). This characteristic allows us to identify putative target genes of a known miRNA sequence by investigating sequence information within a transcriptome or even genome. Using the gametophyte transcriptome generated in the Dolan lab and the 21bp long miRNA sequence information of MpFRH1, TargetFinder (<https://github.com/carringtonlab/TargetFinder>) identified 7 predicted target genes including MpRSL1 (Table 2.1, Honkanen et al., 2018). All predicted target genes were compared to the transcriptome uploaded to phytozome12 (Bowman et al., 2017) and naming was carried out according to their given Mapoly number. The presence of a putative MpFRH1 binding site within the MpRSL1 mRNA is consistent with the hypothesis of MpFRH1 being a negative regulator of MpRSL1 to control the development of

Table 2. 1 Predicted target genes of MpFRH1 within *M. polymorpha* transcriptome.

Target Finder predicts 7 target genes of MpFRH1. Gene names are given according to *M. polymorpha* genome (Bowman et al, 2017), followed by their most significant Arabidopsis orthologs and their gene family. Lower scores indicate a more likely target based on sequence similarity between the miRNA and the target transcript.

MarPoly	Arabidopsis ortholog	Gene family	Score
Mapoly0075s0041	AT5G65950	Foie gras domain containing protein	2.5
Mapoly0011s0195 (MpSSR16)	AT4G34620	Ribosomal protein S16	3.5
Mapoly0053s0025 (MpGMD1)	AT5G66280	GDP-D-mannose 4,6-dehydratase	4
Mapoly0005s0120	AT1G63000	Nucleotide-rhamnose synthase/epimerase- reductase	4
Mapoly0100s0033	AT4G37850	Basic helix loop helix family protein	4
Mapoly0035s0025 (MpSAUR39)	AT3G43120	SAUR LIKE	4
Mapoly0039s0003 (MpRSL1)	AT5G37800	Basic helix loop helix family protein	4

specialised epidermal structures. However, a functional interaction of those two regulators has not been shown yet and the impact of the other putative target genes has not been tested.

This chapter describes the investigation of the 7 predicted target genes including a brief literature survey of their known function in the angiosperm *A. thaliana*. I quantified their relative abundance in MpFRH1^{GOF} compared to the wildtype accession Tak-1 using semi quantitative PCR. MpRSL1 and MpSAUR39 showed a reduced amplification, which was confirmed using quantitative real time PCR. To test if those target genes are indeed cleaved at the predicted target site, I performed a 5'RLM RACE assay followed by sequencing analysis of the PCR product. After detailed analysis MpRSL1 could be confirmed as a target gene of MpFRH1 while none of the other candidate targets were confirmed to be directly targeted by MpFRH1.

To test if an MpFRH1 directed suppression of MpRSL1 is responsible for the rhizoid-less phenotype in MpFRH1^{GOF}, I transformed these mutants with an MpRSL1 transgene that is resistant to MpFRH1 miRNA binding. The restoration of rhizoid and gemmae development in those lines was observed to confirm that 1.) MpFRH1 and MpRSL1 are functionally interacting and 2.) This interaction is indeed controlling the development of rhizoids and gemmae in *M. polymorpha*.

2.3 Results

2.3.1 MpRSL1 and MpSAUR39 transcripts are less abundant in MpFRH1^{GOF} lines

MpFRH1 is a novel miRNA identified in the liverwort *M. polymorpha*, controlling the development of rhizoid cells. The direct targets of MpFRH1 however are not known. Based on TargetFinder seven predicted target genes of MpFRH1 were identified within the *M. polymorpha* transcriptome (Honkanen et al., 2018). TargetFinder aligns a miRNA sequence (21bp) to a query transcriptome and converts them into RNA duplexes. Those duplexes are scored based on their alignment and position of mismatches. Mismatches, single-nucleotide gaps or single-nucleotide bulges are assessed a penalty of +1. G:U base pairs are assessed a penalty of +0.5 while penalty scores are doubled at positions 2-13 relative to the 5' end of the small RNA query sequence. RNA duplexes with a higher penalty score than 4 were rejected (Fahlgren and Carrington, 2010). This analysis (performed by Mario A. Arteaga-Vazquez, Universidad Veracruzana, Mexico) revealed seven target sites and the transcript corresponding to the target site was blasted to the *A. thaliana* genomes to identify the closest orthologs. The transcripts of *M. polymorpha* and their orthologs in *A. thaliana* are listed in table 2.1 (Introduction). To test if the transcript abundance of those genes differs when the miRNA MpFRH1 is more abundant, I tested their transcript abundance in 16-day-old gemmalings of MpFRH1^{GOF} lines using semi quantitative PCR (Figure 2.1). The transcripts of MaPoly0075s0041, Foie Gras Domain Containing Protein, and Mapoly0053s0025, GDP-D-mannose 4,6-dehydratase, were low abundant in Tak-1 plants and MpFRH1^{GOF} plants indicating they are not differently expressed and not suppressed through MpFRH1 at this developmental stage. The biological function of

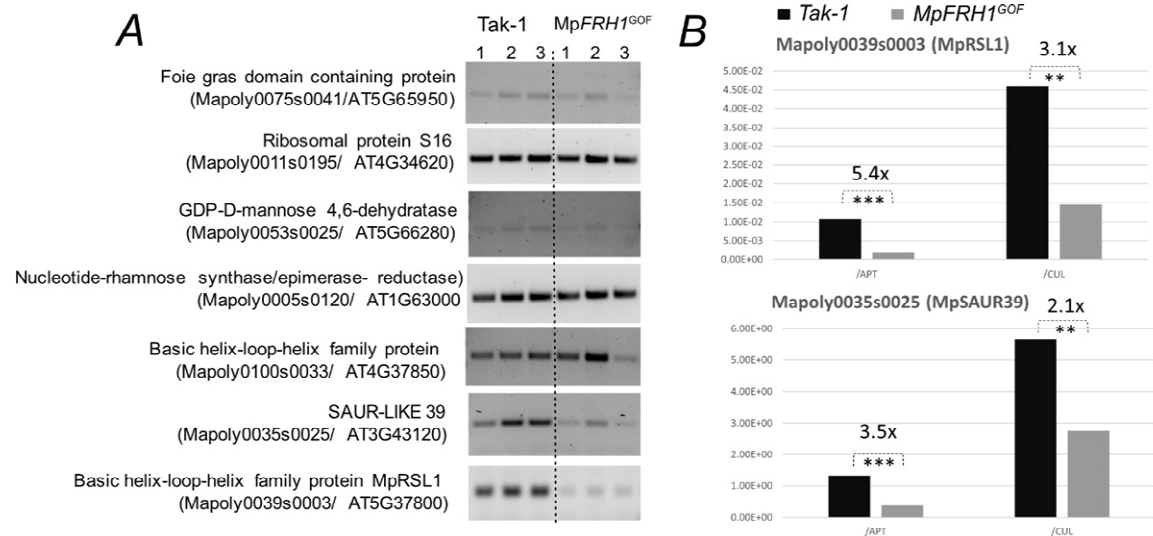


Figure 2.1 Transcript abundance of predicted target genes of MpFRH1

A) Semiquantitative PCR on the 7 predicted target genes in three biological replicates of Tak-1 and MpFRH1^{GOF}. B) Quantitative RT-PCR on Mapoly0035s0025 and Mapoly0039s0003 using MpAPT (left) and MpCUL (right) as housekeeping genes in Tak-1 (black) and MpFRH1^{GOF} (grey). Number given above bars: x-times fold change of mRNA abundance. **=p < 0.01, *** = p < 0.001 (student t-test).

MaPoly0075s0041 is not known to this point and no functional information of its ortholog in *A. thaliana* has been reported. Mapoly0053s0025 is a GDP-mannose 4,6-dehydratase, hence it is likely to participate in fructose and mannose metabolism and not controlling directly the development of specialised epidermal cells. The transcripts of Mapoly0011s0195, Mapoly0005s0120 and Mapoly0100s0033 are more abundant in both Tak-1 and MpFRH1^{GOF} compared to Mapoly0075s0041 and Mapoly0053s0025, but show no different amplification pattern between those genotypes, indicating that the expression of those genes is not directly suppressed through MpFRH1 either (at the developmental stage studied). Mapoly0011s0195s closest ortholog is a Ribosomal protein S16, hence it is likely originated from mitochondrial or chloroplast DNA and involved in translational processes. Mapoly0005s0120 is a dTDP-4-dehydrorhamnose reductase and it is involved in nucleotide sugar metabolism. A function directly linked to cell differentiation for

Mapoly0011s0195 and Mapoly0005s0120 is therefore unlikely. Mapoly0053s0025 is a bHLH TF with its closest homolog in Arabidopsis being At4G37850 (AtbHLH025, Tominaga-Wada et al., 2011). The promoter of this TF is not active in either roots of 5-day-old seedlings or 2-weeks old leaves, suggesting that this bHLH TF does not contribute to root hair or trichome development. There is no evidence suggesting that Mapoly0053s0025 is involved in rhizoid development.

The transcript abundance of Mapoly0035s0025 (MpSAUR39) and Mapoly0039s0003 (MpRSL1), in contrast, are much less abundant in MpFRH1^{GOF} lines compared to Tak-1 suggesting that those two genes are directly or indirectly targeted by MpFRH1. To confirm that MpSAUR39 and MpRSL1 are less abundant in MpFRH1^{GOF} lines I quantified the transcript abundance of those two genes using quantitative real time PCR. I used 2 different housekeeping genes based on Saint-Marcoux et al. (2015), ADENINE PHOSPHORIBOSYL TRANSFERASE 1 (MpAPT1) and CULLIN 1 (MpCUL1) to quantify the relative abundance of MpSAUR39 and MpRSL1. MpSAUR39 is 2.1-3.5 times and MpRSL1 is 3.1 -5.4 times less abundant in MpFRH1^{GOF} lines compared to Tak-1 (relative to MpCUL1 and MpAPT1, respectively). These results are consistent with the previous findings of the semi quantitative PCR and suggest that MpSAUR39 and MpRSL1 are directly or indirectly suppressed by MpFRH1.

In summary, these results show that transcripts of MpSAUR39 and MpRSL1 are less abundant in 16-day-old MpFRH1^{GOF} gemmalings while the other 5 putative targets are not differently abundant at this developmental stage. These findings suggest that MpSAUR39 and MpRSL1 are directly or indirectly targeted by the MpFRH1 miRNA. The other 5 predicted targets do not seem to be affected by MpFRH1 activity – however they were only tested at one developmental stage and the possibility that MpFRH1 activity affects their transcript abundance at other stages during development cannot be excluded.

2.3.2 MpRSL1 mRNA is directly targeted by MpFRH1

MpSAUR39 and MpRSL1 mRNA are less abundant in MpFRH1^{GOF} suggesting that those mRNAs are targets of MpFRH1. To test if MpSAUR39 and MpRSL1 are directly targeted by MpFRH1, I investigated the sequence of the MpFRH1 binding site within MpSAUR39 and MpRSL1 transcript. The putative target site found by TargetFinder that corresponds to MpSAUR39 includes two substitutions and one deletion and is located on the antisense strand (Figure 2.2B). The putative target site corresponding to MpRSL1 is located within an exon of the sense strand approximately 150 bp upstream of the bHLH domain sequence and includes three substitutions (Figure 2.2A). To test if miRNA MpFRH1 targets MpSAUR39 and MpRSL1 and causes a cleavage at the predicted target sites I performed a 5' RLM RACE assay. MiRNA processing activity on cognate target mRNAs generates 3' end cleaved products that are relatively stable and contain ligation-competent, 5'-monophosphate ends rather than conventional 5' caps. This feature makes it possible to ligate directly an RNA oligonucleotide adapter to the 5' terminus of the 3' end cleavage product. The ligated RNA fragments can be reversely transcribed and PCR amplified using gene specific primer (see methods). Amplicons can be sequenced to verify the nature of the sequence and where the transcript terminates, revealing the cleavage site of the target sequence.

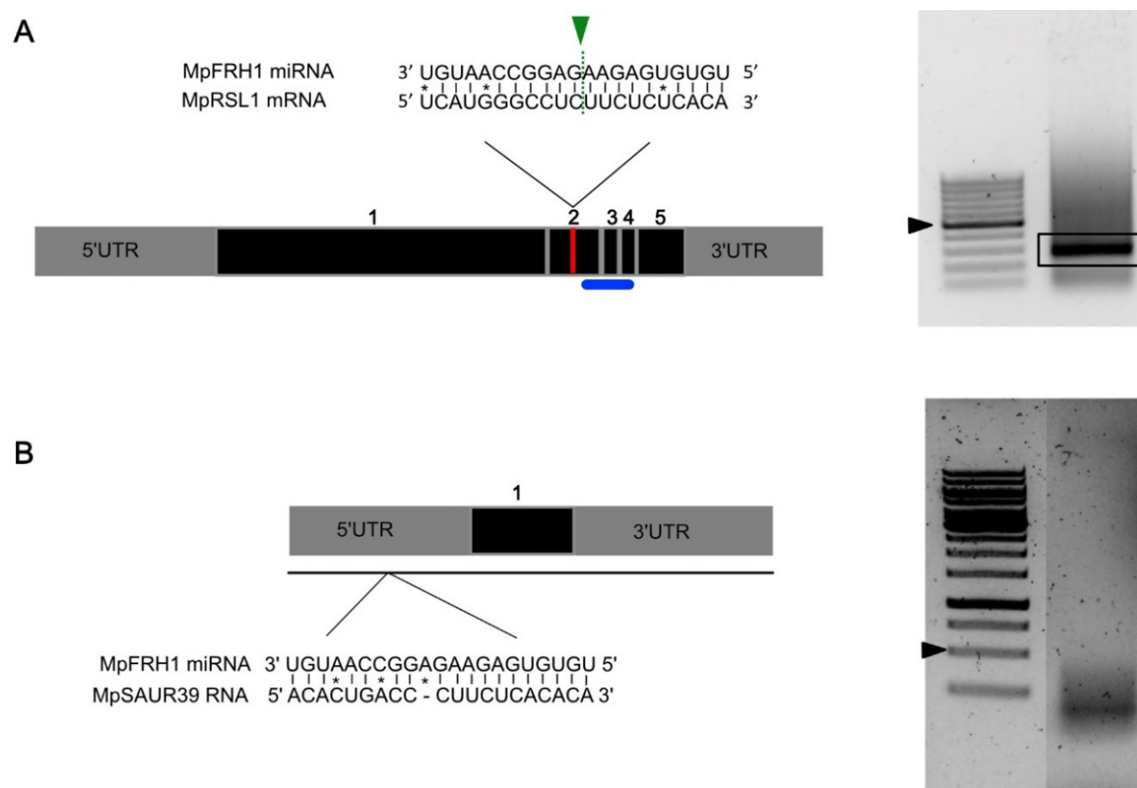


Figure 2. 2 5'RLM RACE PCR on MpRSL1 and MpSAUR39

A) Schematic representation of MpRSL1 transcript with 5' and 3' UTR in grey, 5 exons in black, the bHLH domain underlined in blue, the predicted MpFRH1 target site on the MpRSL1 mRNA highlighted in red with sequence above the transcript. Green arrowhead highlights the predicted cleavage site of MpRSL1 mRNA. 5' RLM RACE assay resulted in a clear band around 250 bp (gel picture to the right) B) Schematic representation of MpSAUR39 transcript with 5' and 3' UTR in grey and exon in black. The predicted MpFRH1 target site is located on the antisense strand and its sequence is given below the transcript. 5' RLM RACE assay did not result in a clear amplification (gel picture to the right). Black arrowheads highlight the 500 bp band of the 100 bp ladder (in A) and 1kb ladder (in B).

A clear amplicon was obtained for the MpRSL1 transcript (Figure2.2A). Sequence analysis of the amplicon verified that the amplified gene product correlated to the MpRSL1 sequence, which terminates at the predicted cleavage site (Figure 2.3). These result show that MpRSL1 is a direct target of MpFRH1 miRNA and that MpFRH1 acts post transcriptionally through mRNA cleavage of MpRSL1. There was no amplification of an

RLM-RACE-PCR product for MpSAUR39 (see Figure 2.2B), indicating that no RNA adapter bound to the predicted target site and hence no PCR amplification was possible. Since the RLM-RACE assay per se is working on the basis of MpRSL1, I conclude that MpSAUR39 antisense strand is not cleaved by the MpFRH1 miRNA, and MpSAUR39 is likely to be not a direct target of MpFRH1. However, miRNAs can also convey posttranscriptional silencing via translational inhibition, which cannot be excluded at this point.

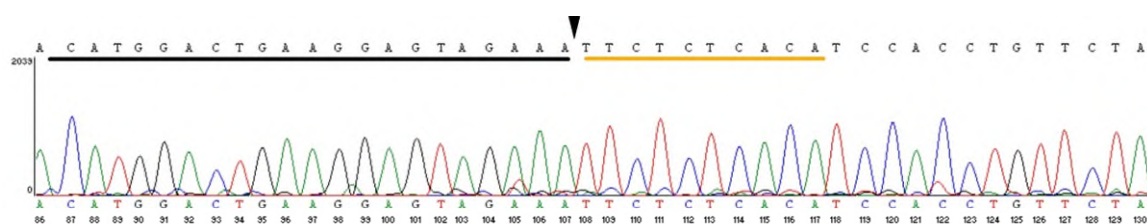


Figure 2. 3 Chromatogram of sequenced MpRSL1 fragment

Black underlined sequence correlates to MpRSL1 mRNA sequence, yellow underlined sequence to the RLM RACE adapter. Transition (black arrowhead) indicated the termination of the MpRSL1 mRNA, which correlated with the predicted MpFRH1 cleavage site on MpRSL1 mRNA.

While the function of MpRSL1 during rhizoid development is well known, there is no direct evidence that MpSAUR39 impacts rhizoid development. In rice (*Oryza sativa*), OsSAUR39 is upregulated by auxin and Kant et al. (2009) reported that OsSAUR39 negatively regulates auxin synthesis and transport. Auxin is well known to affect developmental processes in all plant lineages. In *M. polymorpha* auxin mediated transcriptional regulation acts as a facilitator of branching, differentiation and growth including the formation of rhizoids. Treatment of NAA during gemmaling development resulted in ectopic rhizoid formation (Flores-Sandoval et al., 2015b). If auxin is controlling rhizoid development in *M. polymorpha*, the level of auxin and the expression of auxin related genes are likely to be different in MpFRH1^{GOF} than in wildtype, hence the reduction of MpSAUR39 transcript is likely due to secondary effects of altered auxin levels.

These results show that *MpRSL1* is the only confirmed direct target of *MpFRH1* in developing gemmaling. The other predicted target genes could not be confirmed as direct target genes under the tested conditions. This implies that the phenotypic defects in *MpFRH1^{GOF}* lines are the result of *MpFRH1* directed suppression of *MpRSL1* only, indicating *MpFRH1* as a *MpRSL1* specific miRNA.

2.3.3 Rhizoid development of *MpFRH1^{GOF}* lines can be restored through the insertion of a *MpFRH1* resistant *MpRSL1* transcript

Since *MpRSL1* mRNA was validated as the only direct target of *MpFRH1*, I hypothesise that the developmental defects observed in *MpFRH1^{GOF}* lines, i.e. the lack of specialised epidermal structures like rhizoids, mucilage papillae and gemmae, are due to an ectopic suppression of *MpRSL1*. To test that hypothesis, I generated an *MpRSL1* transgene that is resistant to *MpFRH1* binding (named *MpRSL1^{MUT}*) and transformed *MpFRH1^{GOF}* lines with that transgene (Figure 2.4). Seven point mutations within the 21bp target site, without affecting the amino acid sequence of the *MpRSL1* protein, were introduced using site directed mutagenesis. Hence, the function of *MpRSL1* should not be affected however the mRNA sequence is not complementary to *MpFRH1* anymore and no cleavage should occur. If this is true, *MpRSL1* should be functional and rhizoid development as well as mucilage papillae and gemmae development should be restored. This transgene was expressed under the ubiquitously active *MpEF1 α* promoter. As a control a wildtype *MpRSL1* transgene (named *MpRSL1^{WT}*) was expressed under the same promoter to exclude any dosage effect. Since those lines (*MpFRH1^{GOF};proMpEF1 α :MpRSL1^{WT}*) are still sensitive to *MpFRH1* activity, *MpRSL1* levels are predicted to be suppressed and development of specialised epidermal cells still affected, and hence the phenotype is likely to be similar to *MpFRH1^{GOF}*.

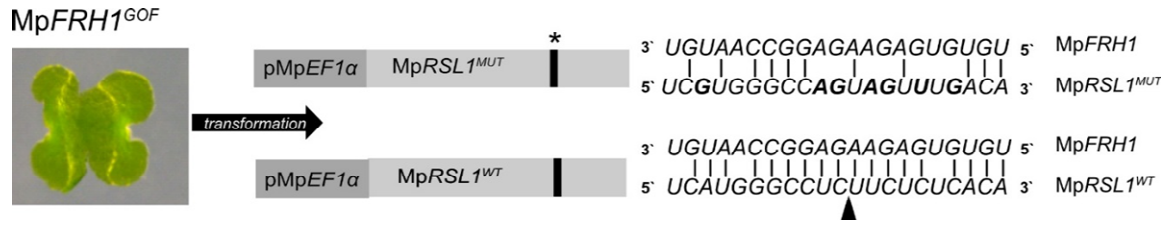


Figure 2. 4 Transformation of $\text{proMpEF1}\alpha:\text{MpRSL1}^{\text{MUT}}$ and $\text{proMpEF1}\alpha:\text{MpRSL1}^{\text{WT}}$ into $\text{MpFRH1}^{\text{GOF}}$

2-week old thalli of $\text{MpFRH1}^{\text{GOF}}$ were transformed with an MpFRH1 resistant MpRSL1 transgene by introducing point mutations within the MpFRH1 target site (black bar, mutated construct marked with an *). Point mutations are shown to the right. While in wildtype there are 18 complementary nucleotides, only 11 remain in $\text{MpRSL1}^{\text{MUT}}$. AA sequence is not affected by those nucleotide mutations. Arrowhead indicates the predicted MpFRH1 cleavage site.

$\text{proMpEF1}\alpha:\text{MpRSL1}^{\text{MUT}}$ and $\text{proMpEF1}\alpha:\text{MpRSL1}^{\text{WT}}$ were transformed into $\text{MpFRH1}^{\text{GOF}}$ using thallus transformation. Thalli incubated with *Agrobacteria* were grown on growth medium including chloramphenicol to select for transformants. Surviving plantlets were then genotyped using a primer pair that amplifies the MpRSL1 transgene and genomic MpRSL1 allele at the same time. Since the genomic MpRSL1 allele still harbours an intron the amplicon has a length of 678bp while the transgene does not harbour any introns and its expected length is 303bp (Figure 2.5).

In total I could extract 26 $\text{MpFRH1}^{\text{GOF}}$ lines harbouring a $\text{proMpEF1}\alpha:\text{MpRSL1}^{\text{WT}}$ transgene and 10 $\text{MpFRH1}^{\text{GOF}}$ lines with a $\text{proMpEF1}\alpha:\text{MpRSL1}^{\text{MUT}}$ transgene. I determined the rhizoid development of those T1 plants and found 25 lines (96%) of the $\text{proMpEF1}\alpha:\text{MpRSL1}^{\text{WT}}; \text{FRH1}^{\text{GOF}}$ lines that developed only few rhizoids similar to $\text{MpFRH1}^{\text{GOF}}$. One line (4%) started to develop visible rhizoids. This indicates that expression of the wildtype version of MpRSL1 from the $\text{proMpEF1}\alpha$ promoter does not suppress the rhizoidless phenotype of $\text{MpFRH1}^{\text{GOF}}$. In comparison, only one $\text{proMpEF1}\alpha:\text{MpRSL1}^{\text{MUT}}; \text{MpFRH1}^{\text{GOF}}$ line was rhizoidless, similar to $\text{MpFRH1}^{\text{GOF}}$, while

seven lines clearly developed rhizoids, and two even developed ectopic rhizoids with rhizoid on their dorsal and ventral side (Figure 2.6).

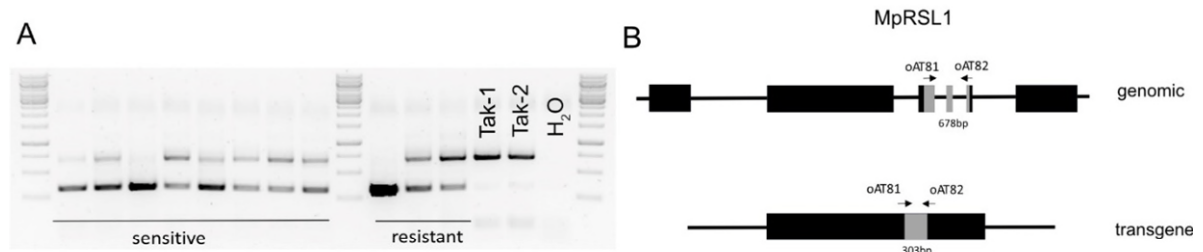


Figure 2. 5 Genotyping of *proMpEF1α:MpRSL1^{WT/MUT}*; *MpFRH1^{GOF}*

A) Agarose gel (1%) electrophoresis of PCR amplified products of *MpRSL1* gDNA and tDNA using oAT81/82 in *proMpEF1α:MpRSL1^{WT}*; *MpFRH1^{GOF}* (left, sensitive) and *proMpEF1α:MpRSL1^{MUT}*; *MpFRH1^{GOF}* (right, resistant) lines. In Tak-1 and Tak-2 only the gDNA allele of *MpRSL1* gets amplified while transformed plants also harbour an intronless *MpRSL1* transgene (doubleband) B) *MpRSL1* gDNA with exons and introns and tRNA without introns. oAT81 and pAT82 amplify a 678 bp long *MpRSL1* gDNA fragment and a 303 bp long tDNA fragment

These findings indicate that rhizoid development of *MpFRH1^{GOF}* lines can be restored by introducing an *MpRSL1* transgene that is resistant to *MpFRH1* miRNA binding, while rhizoid development is still defective in *MpFRH1^{GOF}* lines harbouring an additional *MpRSL1* transgene that is still sensitive to *MpFRH1* binding. This indicates that ectopic expression of a sensitive version is not sufficient to restore rhizoid development (and *MpRSL1* is likely to still be suppressed by *MpFRH1*) while ectopic expression of a resistant version is sufficient. Together these results suggest that the rhizoidless phenotype in *MpFRH1^{GOF}* lines is due to the suppression of *MpRSL1* function. Moreover, this suggests that none of the other target genes are required to restore rhizoid development.

A



B

	no/few rhizoids	rhizoids	ectopic rhizoids	full gemma cups	few gemmae in gemma cup	no gemma cups
$\text{proMpEF1}\alpha:\text{MpRSL1}^{\text{WT}};\text{MpFRH1}^{\text{GOF}}$	25	1	0	0	12	0
$\text{proMpEF1}\alpha:\text{MpRSL1}^{\text{MUT}};\text{MpFRH1}^{\text{GOF}}$	1	7	2	3	2	5

Figure 2. 6 Rhizoid formation in T1 of $\text{proMpEF1}\alpha:\text{MpRSL1}^{\text{WT/MUT}};\text{MpFRH1}^{\text{GOF}}$.

A) Example of a mutant plant that develops few rhizoids only (here $\text{proMpEF1}\alpha:\text{MpRSL1}^{\text{WT}};\text{MpFRH1}^{\text{GOF}}\#2.2$). A second example of a mutant plant that develops rhizoids similar to wildtype (here: $\text{proMpEF1}\alpha:\text{MpRSL1}^{\text{MUT}};\text{MpFRH1}^{\text{GOF}}\#2.7$) and a third example of a mutant plant that develops ectopic rhizoids (here: $\text{proMpEF1}\alpha:\text{MpRSL1}^{\text{MUT}};\text{MpFRH1}^{\text{GOF}}\#2.13$); scale bar=2 mm

B) Quantifications of rhizoid and gemma cup formation in $\text{proMpEF1}\alpha:\text{MpRSL1}^{\text{WT/MUT}};\text{MpFRH1}^{\text{GOF}}$

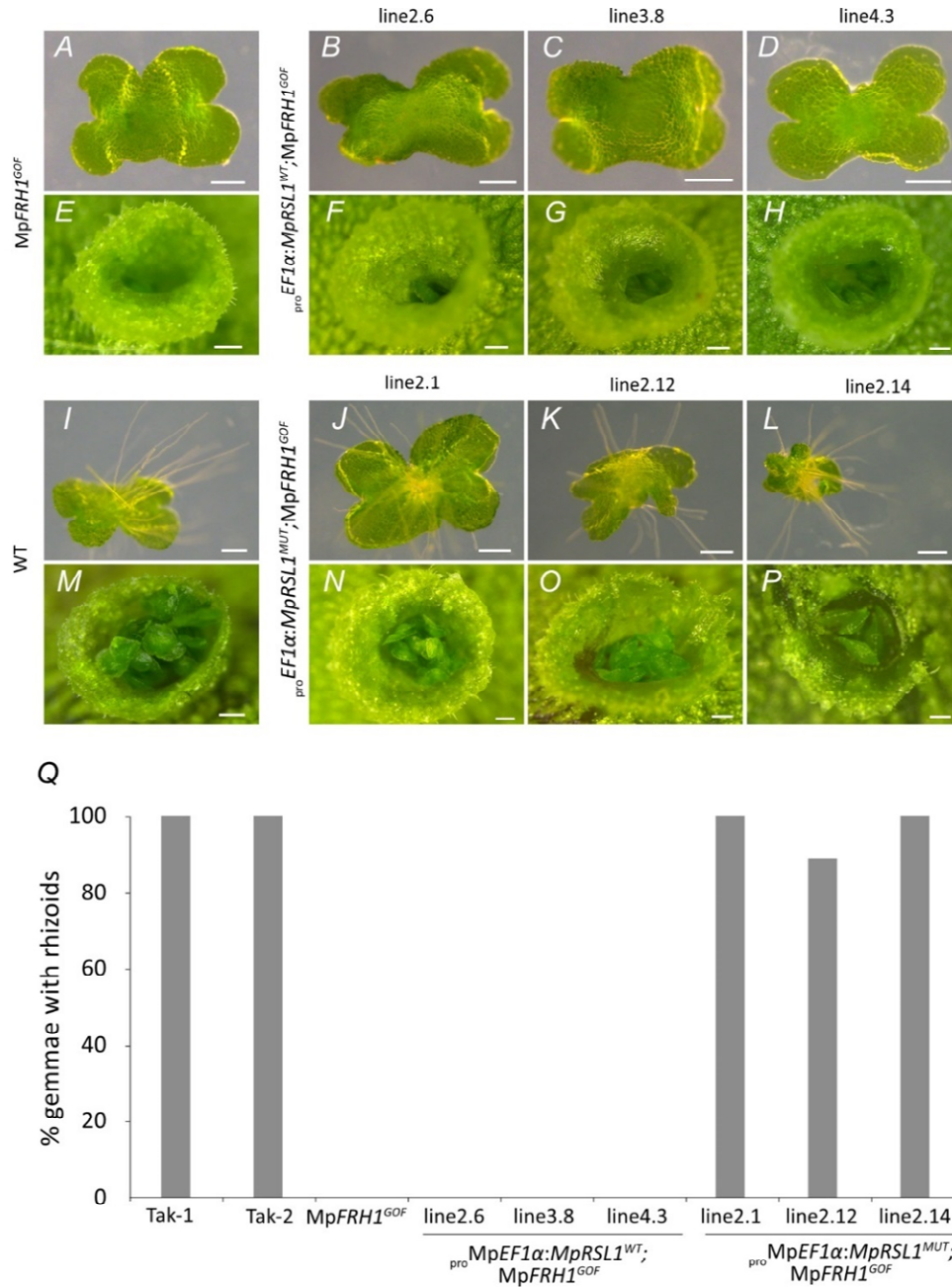


Figure 2. 7 Rhizoid and gemmae formation in wildtype (WT), *MpFRH1*^{GOF} and *proMpEF1α:MpRSL1*^{WT/MUT}; *MpFRH1*^{GOF}

A-D, I- L) 3-day-old gemmalings of *MpFRH1*^{GOF} (A), *proMpEF1α:MpRSL1*^{WT}; *MpFRH1*^{GOF} (B-D), WT (I) and *proMpEF1α:MpRSL1*^{MUT}; *MpFRH1*^{GOF} (J-L)

E-H, M-P) gemma cups of mature *MpFRH1*^{GOF} (E), *proMpEF1α:MpRSL1*^{WT}; *MpFRH1*^{GOF} (F-H), wildtype (M) and *proMpEF1α:MpRSL1*^{MUT}; *MpFRH1*^{GOF} (N-P)

Q) Quantification of 3-day-old gemmalings with more than 3 rhizoids (in %) in wildtype (Tak-1 and Tak-2), *MpFRH1*^{GOF} and *proMpEF1α:MpRSL1*^{WT/MUT}; *MpFRH1*^{GOF}

To verify these results, I randomly selected three independent T1 lines of each genotype and scored for rhizoid formation on 3-day-old gemmalings. The $\text{proMpEF1}\alpha\text{:MpRSL1}^{MUT}$ in MpFRH1^{GOF} plants with both dorsal and ventral rhizoids never developed gemmae and were therefore excluded from further analysis. None of the gemmalings expressing the wildtype MpRSL1 in the MpFRH1^{GOF} background ($\text{proMpEF1}\alpha\text{:MpRSL1}^{WT}$; MpFRH1^{GOF}) developed rhizoids (0% for all three independent lines, Figure 2.7). In contrast, most gemmalings expressing the miRNA resistant form of the MpRSL1 ($\text{proMpEF1}\alpha\text{:MpRSL1}^{MUT}$; MpFRH1^{GOF}) developed rhizoids (100%, 88.9%, 100% for the three independent lines respectively, figure 2.7). The restoration of rhizoid development in $\text{proMpEF1}\alpha\text{:MpRSL1}^{MUT}$; MpFRH1^{GOF} plants demonstrate that MpRSL1 is a target of MpFRH1 in early rhizoid development of young gemmae.

Another phenotypic defect in MpFRH1^{GOF} plants is a strong reduction of gemmae production within gemma cups. To test if also gemmae production is restored in $\text{proMpEF1}\alpha\text{:MpRSL1}^{MUT}$; MpFRH1^{GOF} plants, I investigated the gemma cups of matured thalli. In the T1 of $\text{proMpEF1}\alpha\text{:MpRSL1}^{WT}$; MpFRH1^{GOF} , all lines propagates (in total 12, the others were discarded before maturation stage was reached) developed only few gemmae in their gemma cups (Figure 2.6B, Figure 2.7. F-H) similar to MpFRH1^{GOF} mutants (Figure 2.7, E). In the T1 of $\text{proMpEF1}\alpha\text{:MpRSL1}^{MUT}$; MpFRH1^{GOF} plants, 3 lines developed gemma cups filled with gemmae (10 in total, Figure 2.6, B, Figure 2.7 N-P). Five $\text{proMpEF1}\alpha\text{:MpRSL1}^{MUT}$; MpFRH1^{GOF} mutant lines did not develop gemma cups and two $\text{proMpEF1}\alpha\text{:MpRSL1}^{MUT}$; MpFRH1^{GOF} lines did only develop few gemmae in their gemma cups. The restoration of gemmae development in some $\text{proMpEF1}\alpha\text{:MpRSL1}^{MUT}$; MpFRH1^{GOF} lines demonstrates that MpRSL1 mRNA is also a target of MpFRH1 regulation during gemma development.

Together these data are consistent with the hypothesis that MpFRH1 negatively regulates MpRSL1 by mediating the cleavage of the Mp*RSL1* mRNA during the development of structures derived from single epidermal cells in *M. polymorpha* like rhizoids and gemmae.

2.4 Discussion

Within this chapter I confirmed that miRNA MpFRH1 acts as a negative regulator of MpRSL1 during the development of rhizoids and gemmae, structures that develop from single epidermal cells that grow out of the plane of the epidermis in the liverwort *M. polymorpha*.

While the function of *RSL* class I genes in the development of filamentous tip growing cells seem to be conserved among land plants, MpFRH1 is a liverwort specific miRNA and the Mp*RSL1* binding site is 100% conserved in all available (12 in total) sequenced liverwort species (Honkanen et al., 2018). There is no MpFRH1 target site in the *RSL* class I transcripts of the moss *Physomitrella patens*, the lycophyte *Selaginella kraussiana* or the angiosperm *A. thaliana* (Honkanen et al., 2018). Even though no MpFRH1-like miRNA has been identified to control *RSL* class I genes in *A. thaliana*, the homeodomain protein GLABRA 2 (AtGL2) has been shown to be a negative regulator of *RSL* class I transcription. AtGL2 represses *RSL* class I gene transcription by directly binding to their promoter region (Lin et al., 2015). While the function of MpRSL1 in the development of filamentous tip growing cells is conserved among land plants, there are at least two different mechanism to suppress *RSL* class I gene expression. This suggests that the negative regulation of *RSL* class I genes is likely to have evolved independently in different land plant lineages.

Mp*FRH1* promoter is highly active in RIs, cells that give rise to rhizoids (Honkanen et al., 2018). However, MpFRH1 functions as negative regulator of rhizoid development. This suggests that MpFRH1 may function in the same cells in which it is expressed, and temporally regulate MpRSL1 expression. MpFRH1 could provide a mechanism to fine-tune the activity of MpRSL1 during epidermal fate acquisition or cell outgrowth. Alternatively, the function of MpFRH1 miRNA may be non-cell autonomous.

MpFRH1 miRNA may be a mobile signal that moves from RIs to the neighbouring non-RIs, where it inhibits MpRSL1 expression and therefore RI differentiation. To investigate the function of the miRNA MpFRH1 during rhizoid development the next two chapters provide a detailed description of the function of MpFRH1 during early *M. polymorpha* development (chapter 3) and first insights into the molecular mechanisms whereby MpFRH1 controls MpRSL1 expression at a cellular level (chapter 4).

2.5 Material and Methods

2.5.1 Plant material and RNA extraction

M. polymorpha accessions Tagaragaike-1 and Tagaragaike-2 (Tak-1 and Tak-2), originally from Kobe, Japan) were used as wildtype plants. MpFRH1^{GOF} lines were generated by Suvi Honkanen (Honkanen et al., 2018). Plants were grown from gemmae for 16 days on ½ Gamborg, 0.5 g/l MES and 1.3 % Agar (pH 5.7). Plant material, three replicates per genotype, was harvested into 2ml Eppendorf tubes containing a metal bead and kept in liquid nitrogen. Plant material was homogenized using a Tissue Lyser (QIAGEN, Cat No./ID: 85300). Samples were stored in liquid nitrogen upon application of 700 µl TRIzol™ Reagent (Thermo Fisher, Catalog number: 15596026), incubated at 56°C for 10 minutes and then spun down in a centrifuge at 16000 g for 2 minutes. The supernatant was collected in a new tube, following the instructions of the Direct-zol™ RNA Miniprep (Zymo Research, catalog number R2052). 3 µg total RNA were treated with 1 µl TURBO DNase for 30 minutes at 37°C. The reaction was stopped using 3-4 µl Inactivation reagent (TURBO DNase-free™ kit, Catalog number: AM1907). Samples were tested for successful DNA removal performing a conventional PCR on genomic DNA (primer pAT41, oAT58).

1 µg of DNase treated RNA was used for cDNA synthesis in a total reaction volume of 20 µl using Protoscript II reverse transcriptase (NEB, catalog number M0368), oligo(dT)s and Murine RNase Inhibitor (catalog number M0314) following the manufacturer's instructions. For subsequent analysis cDNA was 10 times diluted.

2.5.2 Semi quantitative PCR

Five µl of 1:10 times diluted cDNA was used for amplification reaction according to Phire Hot Start II DNA Polymerase manufacturer's protocol using the 3-step protocol (with 56°C for annealing) in a total reaction volume of 20 µl. Amplification product after 28 cycles was compared by running PCR product on a 1% agarose gel.

2.5.3 Real time PCR

Mp*APT1* and Mp*CUL3* were selected as reference genes (Saint-Marcoux et al., 2015). qRT-PCT was performed in the Applied Biosystems 7300 Real-Time PCR System (Life Technologies) with SensiMix SYBR Hi-ROX Kit (Bioline) using the primer listed below. Each primer pair was tested to amplify a single product and have amplification efficiency of 1.9-2. Each biological replicate sample was run in three technical replicates. qPCR data was first analysed using LinRegPCR v2012.0 (Ruijter et al., 2009). Average N_0 value of the three technical replicates were calculated for each biological replicate sample. Relative mRNA expression levels in each biological replicate sample were then determined by normalizing the N_0 of each replicate sample separately against each of the two reference genes (Mp*APT1* and Mp*CUL3*).

2.5.4 5' RLM RACE

The Mp*RSL1* transcript cleavage product was identified by carrying out a RLM-RACE PCR as described in Llave et al. (2011). In short, RNA oligonucleotide adaptor (CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA) was first ligated to 5' ends of total RNA extracted from 15-day-old wildtype gemmae. The RNA was reverse transcribed into cDNA using Protoscript II reverse transcriptase (New England

Biolabs) in combination with a gene specific primer GSP RSL1 (TCGTTGGAAGGCCAATAGTC). PCR was then carried out using primer ASP-F (CGACTGGAGCACGAGGACACTGA) that anneals onto the reverse transcribed RNA adaptor and MpRSL1 specific primer nested GSP-RSL1 (GCCTTTTCAAGCATGGTGAC). The PCR reaction was diluted 1:100. One µl of the diluted PCR product was used as a template for a second nested PCR, which was carried out using primers nested ASP-F (GGACACTGACATGGACTGAAGGAGTA) and nested GSP-RSL1 2 (CTCTGAGGATCGTTCGCACT). The resulting PCR products were gel purified, cloned into pGEM-T vector (Promega) and sequenced.

2.5.5 Cloning of *proMpEF1α:MpRSL1^{WT}* and *proMpEF1α:MpRSL1^{MUT}*

MpRSL1 coding sequence was amplified from wildtype *M. polymorpha* cDNA and the resulting PCR product recombined into the pCR8/GW/TOPO Gateway entry vector (Invitrogen) as described in Proust et al. (2016). To generate MpFRH1 miRNA resistant version of MpRSL1 (MpRSL1^{MUT}) seven point mutations were introduced in the predicted MpFRH1 miRNA target site by amplifying the cloned MpRSL1 coding sequence first in two fragments with Phusion High-Fidelity DNA Polymerase (New England Biolabs) using M13F (-20) primer GTAAAACGACGGCCAGTG and mutated gene specific reverse primer GTGGATGTCAAACACTACTGGCCCACGAGGATGAGCGCTTTAGAG for the first fragment, and mutated gene specific forward primer CATCCTCGTGGGCCAGTAGTTTGACATCCACCTGTTCTAAGACTG and T7 universal primer TAATACGACTCACTATAGGG for the second fragment. Full length MpRSL1^{MUT} was then constructed by fusing the two fragments in a PCR reaction containing M13F (-20) primer, T7 universal primer and 1:100 dilution of each of the two

gel extracted fragments from the first PCR. The resulting MpRSL^{WT} and MpRSL1^{MUT} fragments were then recombined into plasmid _{pro}MpEF1 α :Gateway:term pMpGWB207.

2.5.6 Thallus transformation

Thallus transformation was carried out according to Kubota et al. (2013). In summary, the apical sides of 2 week old gemmalings were cut off. Thalli were further cut into four pieces and transferred onto ½ Gamborg growth medium containing 1 % sucrose and 1.3 % agar to grow for 3 days. The pellet of 1ml of saturated *Agrobacterium* culture was resuspended in 5ml M51C medium containing 100 uM acetosyringone, and incubated for up to ~6 hours (28°C with shaking). 10-20 regenerated thalli pieces were transferred to 25 ml of M51C medium containing 2 % sucrose and 100 uM acetosyringone and cocultured for three days with 1 ml of acetosyringone treated *Agrobacterium* cultures (at 22 °C, shaking at 130rpm). Thallus fragments were washed with sterile water and placed onto ½ Gamborg growth medium containing 100 ug/ml cefalotaxine and the appropriate selection reagent. Thallus fragments were grown under normal growth conditions till transformants become visible after 3 to 4 weeks.

2.5.7 Genotyping

Using the Phire Plant Direct PCR Kit plants were genotyped using a small plant fragment of about 0.4-0.5 mm in diameter. DNA amplification was carried out following manufactures' instruction using the 3-step protocol.

2.5.8 Primer sequences

The following primer were used for the quantitative real time PCR:

MpFGP_qPCRF1	CACCACGGCTAAGCAATCAA
MpFGP_qPCRR1	AAATACCACTGCGACCTGGA
MpbHLH qPCR F1	CTTCTCACACACCCAACTGC
MpbHLH qPCR R1	TCAGATCGGTTGAGTTCCCC
MpSAUR qPCR F1	GCATGTCCAAGTCGTCCAAG
MpSAUR qPCR R1	GTTGGTGCTGATGACGTACC
MpSSR16 qPCR F1	CTTGGTAGTCTGGCGTGAGA
MpSSR16 qPCR R1	TTCTCAGTAGGCAGAGCTCG
MpNRS qPCR F1	CTCGTGCTCATCAACTACGC
MpNRS qPCR R1	TGGCCTTGGTCTTGGAGTAG
MpGMD1 qPCR F1	AGAGTAGACGATGGAAGGCG
MpGMD1 qPCR R1	CGCCTTTTCTCACACGACAA
MpRSL1_qPCR F1	AGATGAGTCTGGGGCAACC
MpRSL1_qPCR R1	GGATGAGCGCTTTAGAGTGG
MpAPT1 F	CGAAAGCCCAAGAAGCTACC
MpAPT1 R	GTACCCCCGGTTGCAATAAG
MpCUL3 F	AGGATGTGGACAAGGATAGACG
MpCUL3 R	GTTGATGTGGCAACACCTTG

The following primer were used for genotyping *proMpEF1α::MpRSL1^{WT/MUT}*; *MpFRH1^{GOF}*

Scaffold_300 F2 CTACTACACCACCACCATCACC

RB3 CCTGAATGGCGAATGCTAGAG

oAT81_MpRSL1_CDS_exon2 GACACGTCTCCAAACGTGCAGA

oAT82_MpRSL1_CDS_exon4rev CCTTTTCAAGCATGGTGACCAAGTC

Chapter 3

MpFRH1 suppresses MpRSL1 accumulation to control epidermal patterning in *Marchantia polymorpha*

3.1 Abstract

The development of multicellular organisms relies on complex and dynamic gene regulatory networks. Transcription factors (TFs) are regulators in the hubs of these networks, in which their spatiotemporal expression is highly controlled. MiRNAs have been shown to directly control TF activity. MiRNAs suppress the expression of their target genes by binding to their mRNA to either block the translational process or cause the mRNA to be cut and thereby inactivated. The precise interaction between miRNAs and their target TFs during cell differentiation plays crucial roles during development.

In *M. polymorpha* a novel miRNA, FEW RHIZOIDS 1 (MpFRH1), and its target, the mRNA of the TF ROOT HAIR DEFECTIVE SIX-LIKE 1 (MpRSL1) were identified to control the development of rhizoid cells, which constitute the rooting system of those plants (Proust et al., 2016, Honkanen et al., 2018). Here, I describe the generation of 5 independent *Mpfrh1* loss-of-function mutants (*Mpfrh1*^{LOF}) and demonstrate that the patterning of rhizoid initials (RIs) is defective in these mutant plants. In plants lacking MpFRH1 activity, rhizoid cells initiate closer to each other leading to the development of RI clusters. Furthermore, the lack of MpFRH1 causes the formation of supernumerary mucilage papillae cells, which is another specialized cell type originating from the epidermal cell layer. The *Mpfrh1* mutant plants frequently form supernumerary apical meristems.

I conclude that MpFRH1 is essential to restrict MpRSL1 expression to individual epidermal cells to mediate their differentiation into specialised epidermal cells like rhizoid cells or into multicellular structures like mucilage papillae cells. The meristem of *M. polymorpha* constitutes a single apical cell and the development of supernumerary meristems in *Mpfrh1*^{LOF} suggests that MpFRH1 also restricts the meristem initiation or meristem number. These findings are consistent with the hypothesis that MpFRH1 is a negative signal causing lateral inhibition during the development of single epidermal cells into spatial arrays of specialised cells or organs.

3.2 Introduction

Multicellular organisms are composed of specialised cells that carry out unique functions. The specialisation of cells is tightly controlled through the tight regulation of gene expression. The plant epidermis has been widely used as a simple and accessible model to study cell-fate specification, e.g. root hair cells forming from the root epidermis in the angiosperm *A. thaliana* or rhizoid cells from the ventral side of the thallus in the bryophyte *M. polymorpha*. RSL class I TFs are positive regulators of root hair cells in angiosperms as well as of rhizoid growth in bryophytes, illustrating their conserved function in controlling tip-growing cells with a rooting function among land plants (Menand et al., 2007, Proust et al., 2016). It is well known how RSL class 1 TFs (ROOT HAIR DEFECTIVE-6 [RHD6] and RHD6-LIKE-1 [RSL1]) control root hair initiation in the angiosperm *A. thaliana*, where they promote the initiation of root hair growth in root epidermal cells and further activate *RSL* class II gene expression, which controls root hair outgrowth and elongation (Datta et al., 2011, Yi et al., 2010).

RSL class I TFs in *A. thaliana* are negatively regulated through the class IV HD-ZIP protein GLABRA2 (GL2). AtGL2 is preferentially expressed in non-root hair cells (Masucci et al., 1996), where AtGL2 proteins bind to the promoters of RSL class I genes to mediate their transcriptional repression and hence repress root hair initiation (Lin et al., 2015). Interestingly, only epidermal cells growing in the hollow of two underlying cortical cells become root hair cells, while epidermal cells in contact with only one cortical cell remain non-root hair cells (Dolan et al., 1994, Galway et al., 1994), suggesting that an intercellular positional signalling pathway controls the activation of AtGL2. Due to the organisation of cortical and epidermal cells in the Arabidopsis root, this spatial regulation gives rise to files of root hair and non-root hair cells (Datta et al., 2011). While the function of RSL class I genes is conserved among land plants to control the formation of tip growing

rooting cells, GL2 has not been characterised as a conserved negative regulatory gene among land plants (chapter 1.4, Honkanen et al., 2018).

In the previous chapter I showed that the liverwort specific miRNA MpFRH1 acts as a negative regulator of MpRSL1 expression and it is directly targeting MpRSL1 mRNA to cause post transcriptional gene silencing, and hence suppresses rhizoid formation. This implies that, at least, two different mechanisms have independently evolved during land plant evolution to repress RSL class 1 expression. However, how the miRNA mediated suppression of MpRSL1 expression affects rhizoid development is unknown. It is conceivable that MpFRH1 controls rhizoid cell formation and/or rhizoid growth leading to the following scenarios

- 1.) Dorsal and ventral rhizoids grow within the first days of gemmaling development before dorsiventrality is established. After a few days, when dorsiventrality is establishment, rhizoid development is restricted to the ventral side (reviewed in Bowman, 2016). In plants that constitutively express MpRSL1 (in plants transformed with the *pro35S:MpRSL1* construct) rhizoid formation occurs on both the ventral and dorsal side of mature thalli, indicating that MpRSL1 expression in dorsal epidermal cells is sufficient for rhizoid development (Proust et al., 2016). One possible explanation for these data is that MpFRH1 suppresses MpRSL1 expression in dorsal epidermal cells and therefore rhizoid formation in dorsal epidermal cells during gemmaling development. If this hypothesis (hypothesis 1) were true, I would predict that loss of function mutants in MpFRH1 (*Mpfrh1*^{LOF}) would develop ectopic rhizoids on their dorsal side.
- 2.) The promoter activity of MpFRH1 suggests that MpFRH1 is predominantly transcribed in rhizoid initials (RIs, Honkanen et al., 2018). This suggest that MpFRH1 regulates the expression level of MpRSL1 in growing rhizoid cells. If this

hypothesis were true (hypothesis 2), I would predict *Mpfrh1*^{LOF} plants would develop longer or fast-growing rhizoid cells.

- 3.) The negative regulator of RSL class I genes, AtGL2, in *A. thaliana*, represses the development of root hair cells in cells in particular locations in the epidermis. AtGL2 is therefore important for the spatial patterning of root hair and non-root hair cell files. MpFRH1 on the other hand is a liverwort specific negative regulator of RSL class I expression that might reinforce the formation of non-rhizoid cells similar to AtGL2 in *A. thaliana*. Since the *MpFRH1* promoter is expressed in RIs, the *MpFRH1* miRNA might be produced in RIs but act non-cell autonomously to suppress rhizoid formation in adjacent cells. If this hypothesis were true (hypothesis 3), I would predict that *MpFRH1* would act as a mobile signal and the spatial patterning of rhizoid cells would be defective in *Mpfrh1*^{LOF} mutants.

This chapter describes the developmental defects of plants lacking MpFRH1 activity. The analyses of these loss-of-function mutants allow me to infer the role of MpFRH1 activity during rhizoid establishment. First, I generated plants with a deletion within the *MpFRH1* gene spanning 200 bp upstream and downstream of the 21 bp long miRNA sequence using the CRISPR/Cas9- system. After successful identification of 5 independent *Mpfrh1*^{LOF} lines, I investigated the developmental defects of those lines testing the above-mentioned hypotheses. To test the first hypothesis, I observed the thallus formation including rhizoid formation on the dorsal side in mature *Mpfrh1*^{LOF} and wildtype plants. To test if MpRSL1 positively regulates rhizoid growth and is controlled by MpFRH1, I compared rhizoid growth in *Mpfrh1*^{LOF}, *MpRSL1*^{GOF} (generated in a T-DNA mutagenesis screen, Proust et al., 2016) and wildtype. Both hypotheses were rejected. Ultimately, I characterized how RI patterning and the formation of other epidermal structures are altered in *Mpfrh1*^{LOF}, concluding that MpFRH1 mediates positional information to control epidermal patterning.

3.3 Results

3.3.1 The CRISPR/Cas9 system generated 5 varying *Mpfrh1*^{LOF} lines

MpFRH1 is a miRNA that suppresses the accumulation of *MpRSL1* mRNA and hence negatively controls the formation of rhizoid cells in the liverwort *M. polymorpha*. I hypothesise that MpFRH1 controls MpRSL1 to suppress the development of dorsal rhizoid cells (hypothesis 1), or to suppress rhizoid cell elongation during rhizoid cell growth (hypothesis 2), or it suppresses the formation of RIs during gemma development to control spatial patterning of rhizoid cells (hypothesis 3). To test if one of those hypotheses is true, I generated *Mpfrh1*^{LOF} lines and investigated their rhizoid developmental defects. To generate *Mpfrh1*^{LOF} lines, I used the CRISPR/Cas9 system. I first designed a construct encoding a pair of sgRNAs that would generate cuts at two target sites and result in deletion of the DNA between the two sites. The first sgRNA was complementary to a sequence approximately 400 bp upstream (sgRNA1) and the second sgRNA was complementary to a sequence approximately 200 bp downstream (sgRNA2) of the *MpFRH1* miRNA sequence. When Cas9 endonuclease is guided to the two genomic sequences that are complementary to sgRNA1 and sgRNA2, double strand breaks at both sites will be generated, which can cause a deletion of those 600 bp within the *MpFRH1* allele spanning from sgRNA1 to sgRNA2 (see Figure 3.1).

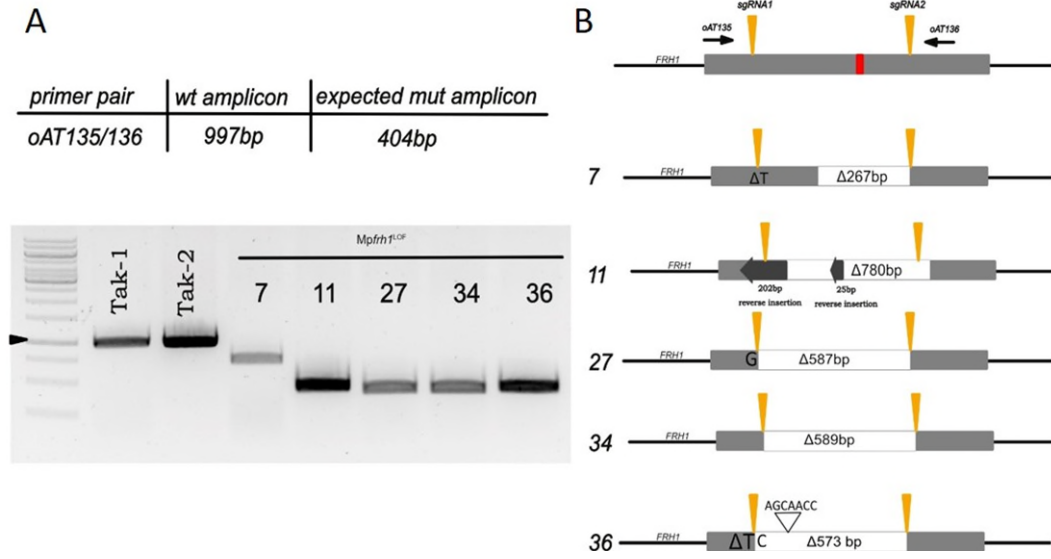


Figure 3. 1 Genotyping and sequence of MpFRH1 alleles in wildtype and Mpfrh1^{LOF7/11/27/34/36}

A) Amplification of MpFRH1 allele using primer oAT135 and oAT136 and its expected length in wildtype (997 bp) and Mpfrh1^{LOF} (404 bp). PCR amplicons of MpFRH1 allele in wildtype (Tak-1 and Tak-2) and Mpfrh1^{LOF} lines loaded onto an agarose gel (1 %, first lane: 1 kb ladder, arrowhead indicates 1 kb band).

B) Diagram of the genomic sequence of the MpFRH1 allele in wildtype and Mpfrh1^{LOFs} with binding sites of primer oAT135 and oAT136, target sites of sgRNA1 and sgRNA2 (yellow triangles) and 21 bp long MpFRH1 miRNA sequence (red). Mpfrh1^{LOF7} with a 267 bp deletion, Mpfrh1^{LOF11} with a 780 bp long deletion and smaller reverse insertions. Mpfrh1^{LOF27/34/36} with deletions of 587 bp, 589 bp and 573 bp deletions respectively and individual SNPs/INDELs in Mpfrh1^{LOF27} and Mpfrh1^{LOF36}.

I transformed *M. polymorpha* spores generated from a cross of Tak-1 and Tak-2 accessions with the transformation vector harbouring the Cas9 endonuclease gene under the expression of the ubiquitously active promoter MpEF1 α , the two sgRNAs within an RNA scaffold under the expression of the U6 RNA Polymerase III promoter and a hygromycin resistance gene. Sporelings were grown for at least 3 weeks on selection media before DNA samples were harvested from hygromycin resistant plants. Of more than 300

plants that were hygromycin resistant and harboured the T-DNA, I tested 36 plants for a deletion in the *MpFRH1* gene. I genotyped the *MpFRH1* gene using primers annealing 200 bp upstream of sgRNA1 and 200 bp downstream of sgRNA2. If there was no deletion within the *MpFRH1* gene, the expected fragment is 997 bp long (e.g. length of wildtype allele). In the event of the Cas9 endonuclease cleaving at sgRNA1 and sgRNA2, I would expect a deletion of around 587 bp and the expected PCR amplicon will be 404 bp long. In total, I obtained 5 lines with a clear deletion within the *MpFRH1* gene. The *MpFRH1* amplicons of line 7, 11, 27, 34 and 36 are shorter than the wildtype amplicon of 1 kb (Figure 3.1). For example, the amplicon of line 7 was 750 bp long and the amplicons of the remaining four lines were approximately 400 bp as expected (Figure 3.1). Sequencing of the PCR product confirmed a deletion around the *MpFRH1* locus including the 21 bp region encoding the *MpFRH1* miRNA sequence in all 5 lines (schematically shown in Figure 3.1., sequencing results in supplementary data). Sequencing the DNA of the *Mpfrh1-7* mutant revealed both a single nucleotide deletion at the sgRNA1 locus and a deletion between a position 267 bp upstream of the sgRNA2 target site and the sgRNA2 target site. The amplicon of *Mpfrh1-11* at the *MpFRH1* locus was the expected 400 bp long. However, sequencing revealed that the *MpFRH1* allele of *Mpfrh1-11* has a deletion of 780 bp between 150 bp upstream of the sgRNA1 target site and 40 bp downstream of the sgRNA2 target site. A 202 bp long fragment, which corresponds to a 202 bp long fragment upstream of sgRNA1 target site till the sgRNA1 target site, was found in reverse direction within the deleted sequence. Another short sequence of 25 bp corresponding to a short fragment of the *MpFRH1* allele between sgRNA1 and sgRNA2 target site was inserted reversely following the 202 bp fragment. The sequence continues to align to the genomic wildtype sequence 40 bp downstream of the sgRNA2 target site. Hence, the overall deletion size was 780 bp but due to the reverse insertion of a fragment of 202 bp

and 25 bp the amplicon is 444 bp long. There was a deletion of 573-587 bp in the *Mpfrh1-27*, *Mpfrh1-34* and *Mpfrh1-36* mutants. This deletion included the sequence between sgRNA1 and sgRNA2 indicating that a double strand break at sgRNA1 and sgRNA2 caused this fragment to be excised from the genome. Individual small nucleotide differences within sgRNA1/2 loci occurred between those lines, which are indicated in Figure 3.1.

Since all lines lack the *MpFRH1* miRNA sequence and no *MpFRH1* activity was expected, they were considered to be loss of function lines and named *Mpfrh1*^{LOF7}, *Mpfrh1*^{LOF11}, *Mpfrh1*^{LOF27}, *Mpfrh1*^{LOF34}, *Mpfrh1*^{LOF36}. These results show that by using the CRISPR/Cas9 system, I was able to generate 5 independent *Mpfrh1*^{LOF} alleles with varying mutations at the *MpFRH1* allele.

3.3.2 *MpRSL1* transcript is more abundant in *Mpfrh1*^{LOF}

The mRNA of *MpRSL1* is a direct target of *MpFRH1* miRNA. To test if the loss of *MpFRH1* causes an increase of *MpRSL1* mRNA abundance, I performed a qPCR analysis and compared the relative *MpRSL1* transcript abundance in *Mpfrh1*^{LOF} to Tak-1 and Tak-2. I harvested 6-day-old gemmalings of each genotype with 3 biological replicates each (*Mpfrh1*^{LOF7} consists of only 2 biological replicates) and extracted their RNA, followed by cDNA synthesis. *MpRSL1* RNA abundance was normalised to the housekeeping gene *MpAPT1* (Saint-Marcoux et al., 2015). In *Mpfrh1*^{LOF7}, *Mpfrh1*^{LOF11} and *Mpfrh1*^{LOF27}, *MpRSL1* mRNA was approximately 1.5 times more abundant than in wildtype (Tak-1 and Tak-2) while *MpRSL1* mRNA is 2-2.5 times more abundant in *Mpfrh1*^{LOF34} and *Mpfrh1*^{LOF36} (see Figure 3.2). These changes indicate that the accumulation of *MpRSL1* mRNA in *Mpfrh1*^{LOF} lines is higher than in Tak-1 and Tak-2 plants. I carried out an independent qPCR assay to quantify *MpRSL1* mRNA abundance in *Mpfrh1*^{LOF} mutants of

7-day-old gemmalings. I was able to observe the same trend, displaying *MpRSL1* mRNA being 1.5 (in *Mpfrh1*^{LOF36}) to 4 times (in *Mpfrh1*^{LOF27}) more abundant than in Tak-1 and Tak-2 plants (see Supplementary Figure S3.1).

These results show that the transcript level of *MpRSL1* in *Mpfrh1*^{LOF} is more abundant than in wildtype plants in 6 to 7-day-old gemmalings. This is consistent with the finding that *MpFRH1* targets *MpRSL1* transcript for cleavage. Altogether, these data show that *MpFRH1* post transcriptionally silences *MpRSL1* and suppresses *MpRSL1* accumulation during early gemmaling development, when rhizoids develop.

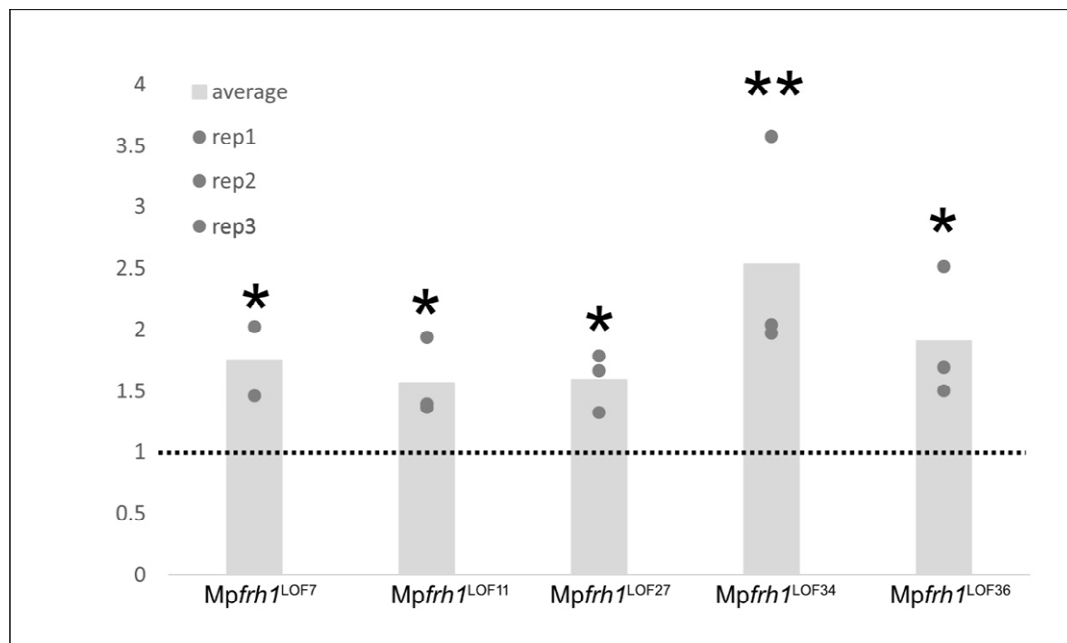


Figure 3. 2 X-fold change of *MpRSL1* mRNA abundance in 6-day-old gemmalings of *Mpfrh1*^{LOF} compared to wildtype (Tak-1/ Tak-2)

Relative *MpRSL1* mRNA abundance in 6-day-old gemmalings in *Mpfrh1*^{LOF} (n=3, only for *Mpfrh1*^{LOF7} n=2) was compared to relative *MpRSL1* mRNA abundance in wildtype (Tak-1 and Tak-2 combined, dotted line, n=6). Difference of *MpRSL1* mRNA abundance given as x-fold times change. Statistical significance based on student t-test is given for each line compared to Tak-1/Tak-2 (combined) *=p < 0.05, ** = p < 0.01.

3.3.3 MpFRH1 does not suppress the development of dorsal rhizoids

MpFRH1 directly targets *MpRSL1* mRNA during early gemmaling development. *MpRSL1* is a positive regulator of rhizoid development. While *Mprsl1^{LOF}* thalli are strongly impaired in their rhizoid development, *MpRSL1^{GOF}* thalli grow rhizoids on their ventral and dorsal epidermis, indicating that *MpRSL1* expression on the dorsal side is sufficient to promote rhizoid formation on the dorsal epidermis (Proust et al., 2016). Gemmae have not established dorsoventrality during gemma development in the gemma cup and rhizoids grow on both sides during early gemmaling development. However after 1-3 days of gemmaling development (day 0 is the time when a gemma is in contact with growth substrate), dorsoventral polarity is established and rhizoid formation is restricted to the ventral side (based on Mirbel, 1835 and Pfeffer, 1871, summarized in Bowman, 2016). Since *MpRSL1* mRNA is the direct target of MpFRH1, I hypothesise that MpFRH1 is important to attenuate *MpRSL1* mRNA accumulation at the dorsal side of the thallus and hence suppresses the emergence of rhizoids on the dorsal side during gemmaling development, during dorsiventrality establishment. If this hypothesis were true, *Mpfrh1^{LOF}* plants would develop rhizoid cells on their dorsal side similarly to the *MpRSL1^{GOF}* lines. To test this hypothesis, *Mpfrh1^{LOF}* plants were grown along Tak-1 and Tak-2 wildtype accessions over 23 days to observe if dorsal rhizoids are produced. In Tak-1 and Tak-2 gemmaling development, starting from a disc shape propagule with a stalk attachment point and two notches at each end (highlighted in Figure 3.3), begins with a lateral expansion of the tissue around the meristematic notches and lobe expansion outwards. Rhizoid development occurred within the first 24 hours (see supplementary data S3.2). After 3-5 days, gemmalings showed elongated lobes with multiple rhizoids on both sides, dorsal and ventral. No further rhizoids developed from the dorsal side of the thallus (Figure 3.3, S3.2 for day 2 and 3). Later, thallus- bifurcation occurred to produce two

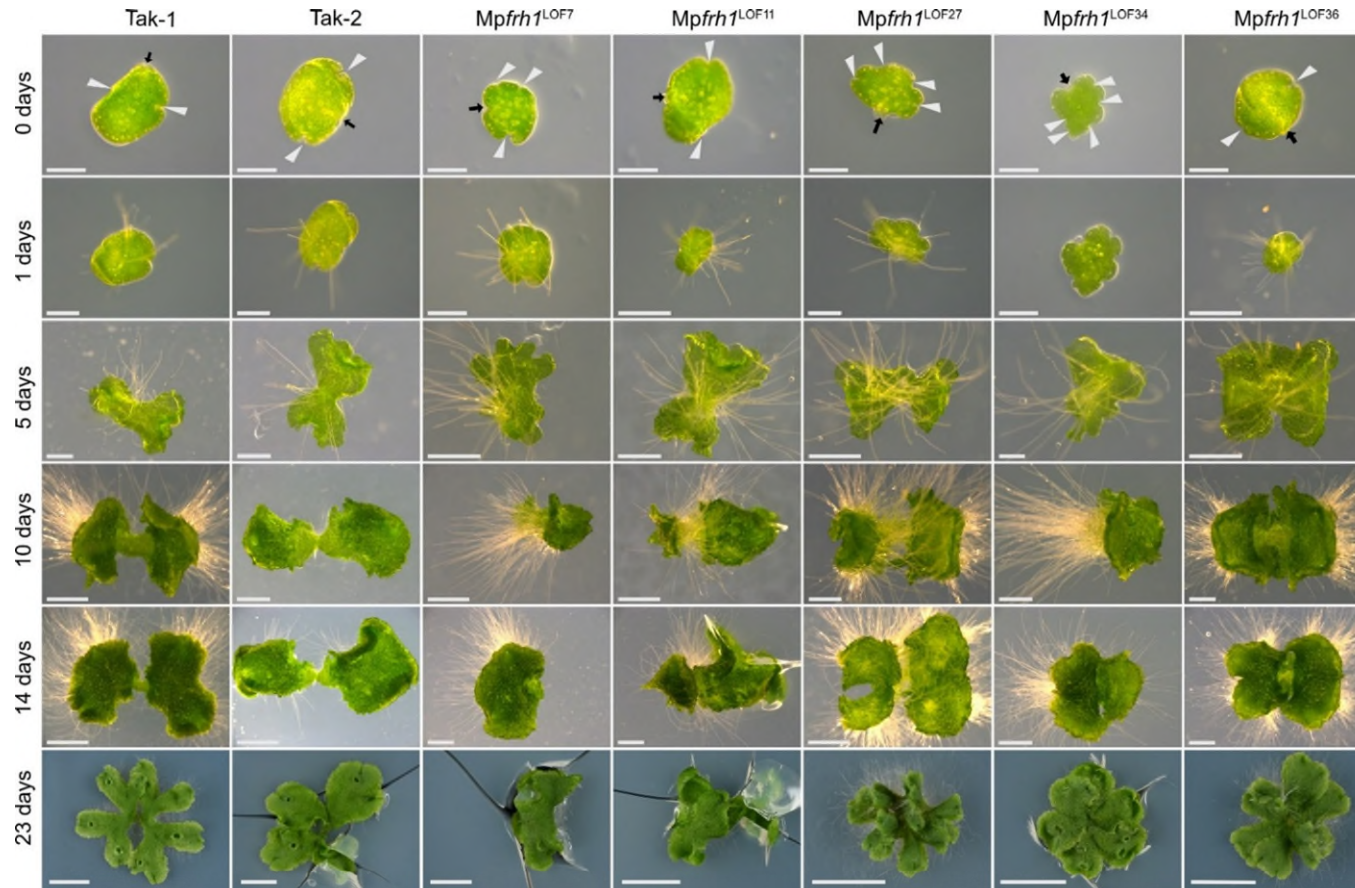


Figure 3. 3 Gemmaling development of *Mpfrh1*^{LOF} and wildtype over 23 days

Gemmaling development of wildtype and *Mpfrh1*^{LOF} starting from 0-day-old gemma to 1, 5, 10, 14 and 23-day-old gemmalings. Arrowheads indicate apical notches, black arrows indicate the stalk. Scale bars : 0 days: 200 μ m; 1 day : 200 μ m (except *Mpfrh1*^{LOF11} and *Mpfrh1*^{LOF36}, here 500 μ m); 5 days : 500 μ m (except *Mpfrh1*^{LOF34}, here 200 μ m), 10 days : 1 mm (except *Mpfrh1*^{LOF27,34,36}, here 500 μ m), 14 days: 2 mm for wildtype, 1 mm for *Mpfrh1*^{LOFs}, 23 days: 1cm.

apical notches on each side of the thallus, which constitute the new growing apices until subsequent bifurcation occurs (Solly et al., 2017). After 23 days a flat clearly bifurcated thallus with gemma cups, a dorsal feature characteristic of mature plants, developed. Unlike in wildtype plants, dorsal rhizoids are clearly visible at this stage in *MpRSL1^{GOF}* plants (Proust et al., 2016). Dorsal rhizoids could not be observed in any of the *Mpfrh1^{LOF}* lines after 23 days (see Figure 3.3). The lack of dorsal rhizoids in *Mpfrh1^{LOF}* after 23 days indicates that the miRNA *MpFRH1* does not suppress the accumulation of *MpRSL1* mRNA at the dorsal side of the thallus to prevent the formation of dorsal rhizoid cells. However, the characteristic bifurcation pattern of Tak-1 and Tak-2 is impaired in *Mpfrh1^{LOF}*, which will be discussed in section 3.3.8/9. Therefore, I reject the hypothesis 1, that *MpFRH1* represses dorsal rhizoid development.

3.3.4 *MpFRH1* does not suppress rhizoid elongation rate

In *A. thaliana* RSL class I TFs (AtRHD6 and AtRSL1) accumulate in cells that will develop a root hair (Menand et al., 2007) where they promote root hair initiation and activate RSL class II TFs (e.g. AtRSL4, AtRSL2, AtRSL3 and AtRSL5; Yi et al., 2010, Pires et al., 2013). RSL class II TFs regulate root hair elongation. While the function of RSL class I TFs is conserved among land plants, the function of RSL class II in *M. polymorpha* remains unknown. In the T-DNA mutagenesis screen of rhizoid defective mutants, the single RSL class II gene (*MpRSL2*) in the *M. polymorpha* genome was not identified as a rhizoid regulator. Furthermore, *MpRSL2* mRNA was not detected in rhizoid cells (Proust et al, 2016, unpublished data). This suggest that *MpRSL2* might play a minor role during rhizoid formation, if any. Possibly *MpRSL1* not only controls rhizoid initiation but also regulates rhizoid growth and rhizoid elongation. If *MpRSL1* is also active during rhizoid elongation, the miRNA *MpFRH1* might be an important repressor of rhizoid elongation to terminate

rhizoid growth by reducing MpRSL1 activity. Since the promoter of MpFRH1 is predominantly active in RIs (Honkanen et al., 2018), it suggests that MpFRH1 is active in RIs and suppresses MpRSL1 activity in those cell types. I hypothesise that MpRSL1 controls rhizoid initiation as well as rhizoid elongation and MpFRH1 controls MpRSL1 mRNA accumulation during rhizoid elongation. If this hypothesis were true, MpRSL1 accumulation in RIs would be enhanced in *Mpfrh1^{LOF}* lines which would increase MpRSL1 activity in those cells resulting in longer rhizoids. Furthermore, *MpRSL1^{GOF}* lines would develop longer rhizoids. To test that hypothesis (hypothesis 2), I measured the maximum length of the rhizoid population of *Mpfrh1^{LOF}*, *MpFRH1^{GOF}*, two independent *MpRSL1^{GOF}* mutants and Tak-1 and Tak-2 plants. Plants were grown from gemmae and the rhizoid elongation was quantified between 14 and 28 days of growth. Tak-2 plants developed the longest rhizoids and after 28 days the rhizoids were 30.1 mm (SD = 4.26). The rhizoids of Tak-1 plants grew less than Tak-2, reaching a mean length of 24 mm (SD = 2) after 28 days (Figure 3.4).

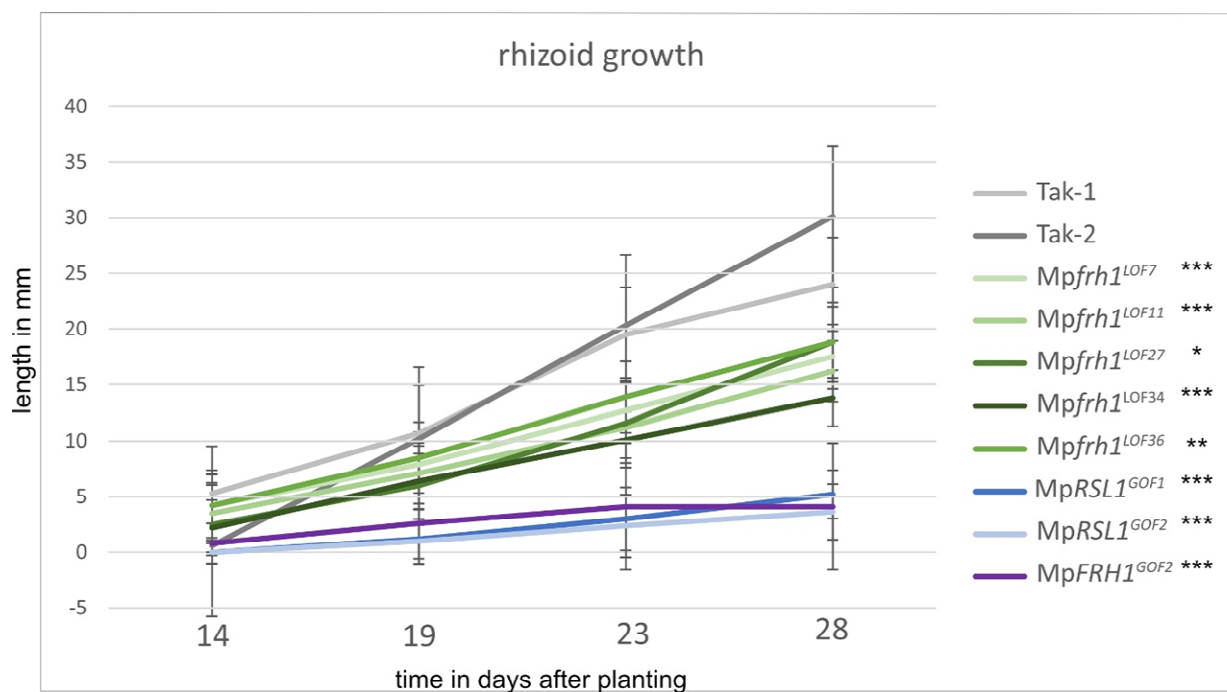


Figure 3. 4 Rhizoid length in wildtype, *Mpfrh1*^{LOF/GOF} and *MpRSL1*^{GOF}

Length of rhizoid population in *Mpfrh1*^{LOF} (green), *MpRSL1*^{GOF} (blue), *MpFRH1*^{GOF} (purple), and Tak-1 and Tak-2 (grey) after 14, 19 , 23 and 28 days of gemmaling growth (n=5). Statitical significance based on student t-test is given for each line compared to Tak-1 at 28 days after planting, *=p < 0.05, ** = p < 0.01, *** = p < 0.001

Mpfrh1^{LOF} were shorter than both Tak-1 and Tak-2. The average length of the rhizoid population in *Mpfrh1*^{LOF7} was 17.5 mm (SD = 1.8) long and in *Mpfrh1*^{LOF11} 16.2 mm (SD =0.76). Rhizoids of *Mpfrh1*^{LOF34} grew to a length of 13.8 mm (SD = 3.19) while *Mpfrh1*^{LOF27} and *Mpfrh1*^{LOF36} grew to a length of 18.8 mm (SD = 3.49 and 2.36, respectively). These results show that rhizoids are shorter in *Mpfrh1*^{LOF} mutants than wildtype (Tak-1 or Tak-2). These data indicate that *MpFRH1* does not repress *MpRSL1*-mediated rhizoid elongation. These results are supported by the findings of the rhizoid growth of *MpRSL1*^{GOF} lines between 14 and 28 days. Rhizoids of *MpRSL1*^{GOF} lines were very short (e.g. for *MpRSL1*^{GOF1} they were on average 5.2 mm, SD = 2.17 and for *MpRSL1*^{GOF2} they were on average 3.63 mm, SD = 2.5) and rhizoid length was very similar to *MpFRH1*^{GOF} lines (e.g. on average 4.1 mm, SD = 5.68). If *MpRSL1* were involved in rhizoid elongation we would expect rhizoid length in *MpRSL1*^{GOF} lines to be longer than in wildtype. Since rhizoid length in either *MpRSL1*^{GOF} or *Mpfrh1*^{LOF} is not longer than in wildtype, I conclude that 1.) *MpRSL1* is not a positive regulator of rhizoid elongation and 2.) *MpFRH1* does not suppress *MpRSL1* expression during rhizoid elongation (Figure 3.4).

Since rhizoids of *MpRSL1*^{GOF} and *Mpfrh1*^{LOF} mutants do not elongate as much as wildtype, these results suggest that *MpRSL1* is not a positive regulator of rhizoid elongation. It also suggests that *MpFRH1* does not reduce *MpRSL1* activity during rhizoid elongation and hence does not act as a suppressor of rhizoid growth. However *MpRSL1*^{GOF}

plants develop more rhizoids overall (Proust et al., 2016, Figure S3.3). The short rhizoid phenotype of *Mpfrh1^{LOF}* and *MpRSL1^{GOF}* might be explained by a compensation of rhizoid number to rhizoid length, which would require further investigation. The short rhizoid phenotype of *MpFRH1^{GOF}* gemmalings can be explained by the fact that those plants hardly develop any rhizoids and hence rhizoid populations occur as non-elongating.

3.3.5 MpFRH1 controls the patterning of RI clusters and affects their cell size

Since lack of MpFRH1 activity neither regulates the formation of dorsal rhizoids (hypothesis 1) nor enhances rhizoid elongation (hypothesis 2), I tested if the lack of MpFRH1 activity has any effect on the density of rhizoids developing on the epidermal surface of gemmae (hypothesis 3). I imaged 0-day-old gemmae, using Laser Confocal Microscopy, and stained the cell walls of epidermal cells using propidium iodide (PI). At the same time chloroplasts were visualised by their autofluorescence; chloroplasts fluoresce in the red part of the spectrum when excited with 543nm. RIs are enlarged cells with smaller chloroplasts than surrounding epidermal cells that barely emit fluorescence after excitation, which makes them easy to identify (highlighted in Figure 3.5). In wildtype gemmae, RIs were relatively equally distributed and individual RIs were separated from each other by epidermal cells (Figure 3.5. A-F). Occasionally they appeared in small groups of two or three adjacent RIs. In *Mpfrh1^{LOF}* more RIs developed next to each other, resulting in the development of more RI clusters than in wildtype (Figure 3.5 G-U). To test if more RIs develop in *Mpfrh1^{LOF}* mutants than in wildtype, I measured the relative area of RIs per gemma. While approximately 5 – 6 % of the gemmae surface were made up of RIs in wildtype (5.07% [SD = 0.53] in Tak-1 and 6.13 % [SD = 0.91] in Tak-2), the area of RIs per gemma in *Mpfrh1^{LOF}* was higher than 6 %. The area of RIs per gemma varied from 7.12 % in *Mpfrh1^{LOF34}* (SD = 1.10) to 11.92 % in *Mpfrh1^{LOF7}* (SD = 1.62, Figure 3.5 V). The larger area of RIs on gemmae in *Mpfrh1^{LOF}* mutants than in wildtype indicates that the lack of MpFRH1 causes either the development of supernumerary RIs on the epidermal surface, or causes an increase in RI surface area.

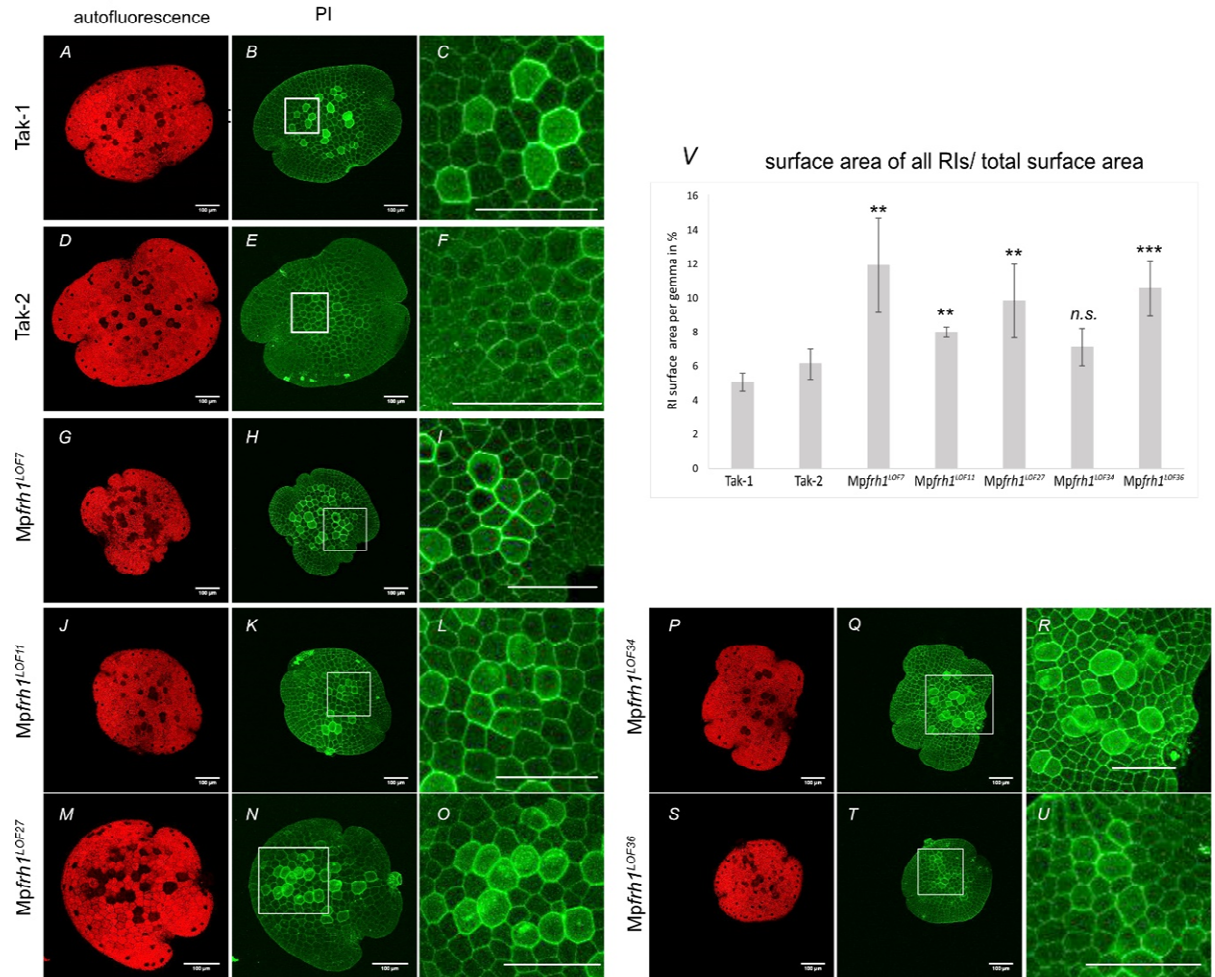


Figure 3.5 Rhizoid patterning in *Mpf1*^{LOF}

Laser Confocal Microscopy shows RIs in Tak-1 and Tak-2 (A-F) and in *Mpf1*^{LOF} (G-U) in 0-day-old gemmae. Autofluorescence of chlorophyll is coloured in red, PI emission coloured in green. Third column shows the enlarged squared region of rhizoid clusters highlighted in second column of each line. Total area (in %) of RIs per gemma in *Mpf1*^{LOF} and Tak-1 and Tak-2 (V, n=3 apart from Tak-2 [n=2] and *Mpf1*^{LOF11} [n=4]). Statistical significance based on student t-test is given for each line above the bars, compared to Tak-1 and Tak-2 (combined), ** = p < 0.01, *** = p < 0.001, n.s. = not significant, p > 0.05

3.3.6 Lack of MpFRH1 expression causes the development of larger RIs

To test if RIs in *Mpfrh1^{LOF}* plants are larger than in wildtype, I quantified the surface area of the outer surface of RIs relative to the overall size of their corresponding gemma. I calculated the combined surface area of the three largest RIs in wildtype and *Mpfrh1^{LOF}* mutants. In Tak-1 and Tak-2 the three largest RIs on a 0-day-old gemma accounted, on average, 0.36 % (SD = 0.05) and 0.33 % (SD = 0.02) of the total surface area. In *Mpfrh1^{LOF}* the largest RIs accounted for about twice this amount. In *Mpfrh1^{LOF7}* and *Mpfrh1^{LOF27}* the three biggest RIs accounted on average 0.74% (SD = 0.21), in *Mpfrh1^{LOF11}* and *Mpfrh1^{LOF36}* 0.66 % (SD = 0.16 and 0.02, respectively) and in *Mpfrh1^{LOF34}* 0.52 % (SD = 0.31) of the total surface. I conclude that the maximal surface area of *Mpfrh1^{LOF}* RIs are larger than in wildtype relative to their respective gemma area, which indicates that MpFRH1 suppresses the increase of RIs surface area. However this could be due to the fact that the overall size of gemmae were smaller in *Mpfrh1^{LOF}* mutants. While wildtype (Tak-1 and Tak-2) gemmae were on average 0.26-0.32 mm² (SD = 0.06), *Mpfrh1^{LOF}* gemmae were only 0.10 to 0.14 mm² (SD = 0.037). Therefore the relatively larger surface area of RIs in *Mpfrh1^{LOF}* might be either due to RI expansion, or due to a secondary effect of gemmae being smaller and consequently the relative RI size is increased (Figure 3.6).

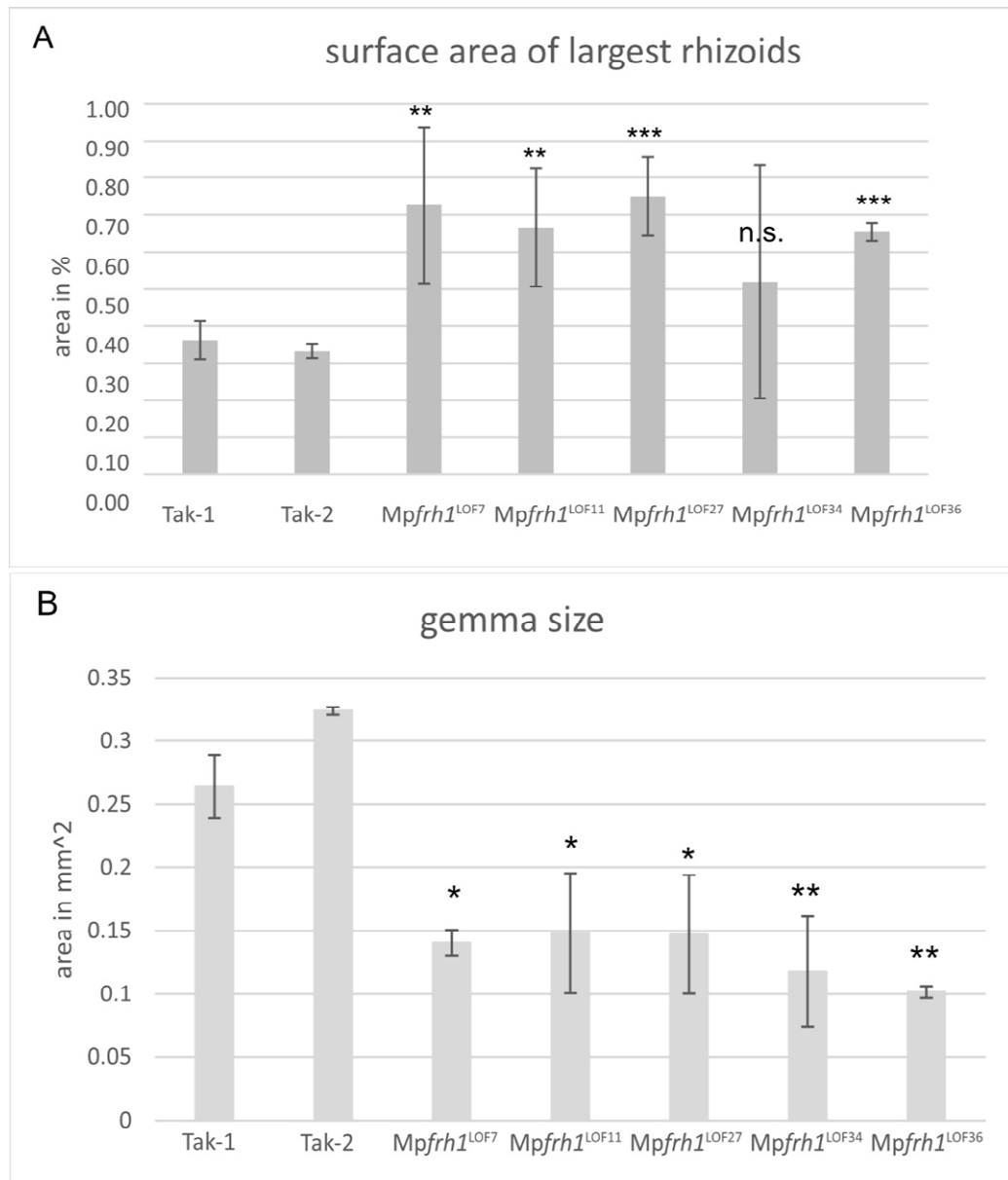


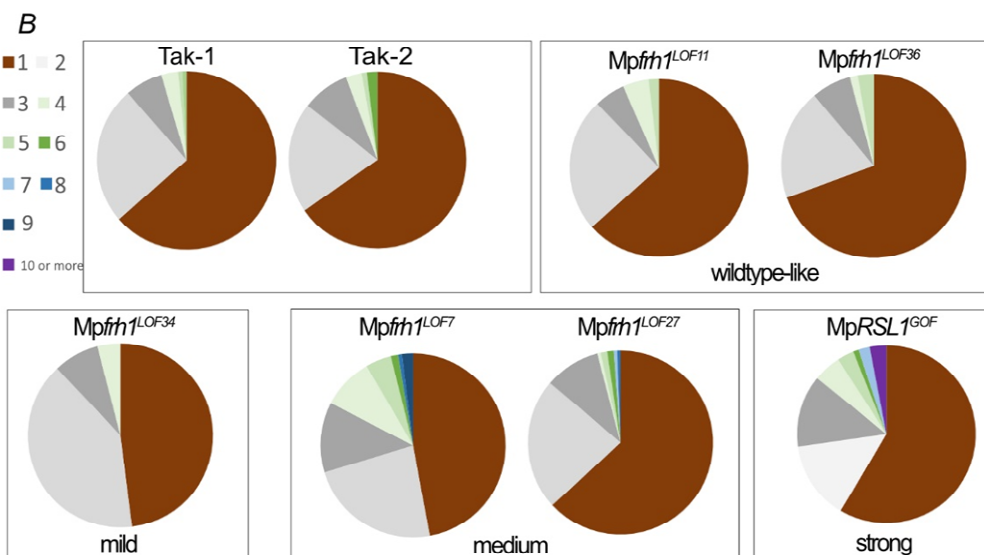
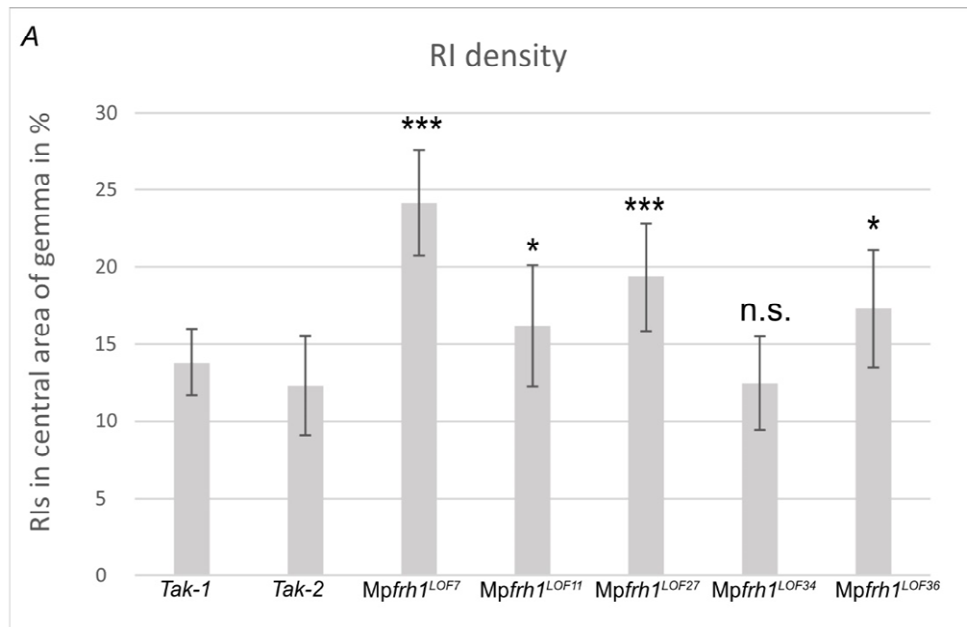
Figure 3.6 Relative size of three largest RIs and gemma size in *Mpfrh1*^{LOF}

Surface area of the three largest RIs in Tak-1, Tak-2 and *Mpfrh1*^{LOF} (in %, A) relative to total area of their gemma. Gemma area of Tak-1, Tak-2 and *Mpfrh1*^{LOF} in mm² (B). n=3 (Tak-2 n=2, *Mpfrh1*^{LOF11} n=4). Statistical significance based on student t-test is given for each line above the bars, compared to Tak-1 and Tak-2 (combined), * = p < 0.05, ** = p < 0.01

3.3.7 MpFRH1 suppresses the development of abundant RIs

The area of RIs developing on *Mpfrh1*^{LOF} gemmae is larger than in wildtype (Tak-1 and Tak-2). One explanation for this is that the surface area of RIs is greater in *Mpfrh1*^{LOF} than in wildtype. An alternative hypothesis is that the number of RIs that develop on the epidermal surface of gemmae is greater in *Mpfrh1*^{LOF} than in wildtype. To test this hypothesis, I quantified the number of RIs that developed in the central region of the gemmae. The central region was defined subjectively for each gemma as the area of fully developed cells, away from the apical notch. On average I sampled 88 cells in the central area of each gemma. However, when cells were located too close to the meristematic notches they were excluded and fewer cells were sampled for that gemma (minimum 53 cells). In Tak-1, 13.5%, and in Tak-2, 12.3%, of the epidermal cells were RIs, in *Mpfrh1*^{LOF7}, *Mpfrh1*^{LOF11}, *Mpfrh1*^{LOF27} and *Mpfrh1*^{LOF36} 17.3 – 24.2% of the epidermal cells were RIs. This indicates that MpFRH1 activity reduces the number of epidermal cells differentiating into RIs during gemmae development (Figure 3.7A)

To investigate how RIs are distributed in the epidermis I investigated the patterning of RIs in wildtype and *Mpfrh1*^{LOF}. First, I quantified the size of each RI cluster, e.g. if a RI develops as a single cell surrounded by epidermal cells or if multiple RIs develop next to each other to form a cluster of 2 or more adjacent RIs. In wildtype (Tak-1 and Tak-2) plants, 63% of RIs were individuals and were surrounded by non-RI cells. No clusters larger than 6 RIs were observed in either Tak-1 or Tak-2. About 20-25% of RIs form a cluster of 2 adjacent RIs, 6.8-8.7% were in clusters of three adjacent RIs and 3% developed in clusters of 4 RIs. Fewer than 3% of clusters comprised 5 or 6 RIs (0.8-1%, 0.8-1.9%, respectively, Figure 3.7 B and C).



C

# of RICS genotype	1	2	3	4	5	6	7	8	9	≥10
Tak-1	63.6	25.0	6.8	3.0	0.8	0.8	0.0	0.0	0.0	0.0
Tak-2	65.4	20.2	8.7	2.9	1.0	1.9	0.0	0.0	0.0	0.0
<i>Mpfrh1</i>^{LOF7}	47.1	23.2	12.3	9.0	4.5	1.3	0.0	0.6	1.9	0.0
<i>Mpfrh1</i>^{LOF11}	63.6	24.3	5.6	4.7	1.9	0.0	0.0	0.0	0.0	0.0
<i>Mpfrh1</i>^{LOF27}	63.2	23.0	9.8	0.6	1.1	1.1	0.6	0.6	0.0	0.0
<i>Mpfrh1</i>^{LOF34}	48.0	40.0	8.0	4.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>Mpfrh1</i>^{LOF36}	69.4	19.4	6.9	1.4	2.8	0.0	0.0	0.0	0.0	0.0
<i>MpRSL1</i>^{GOF}	58.59	14.14	13.13	5.05	3.03	1.01	2.02	0.00	0.00	3.00

Figure 3. 7 Quantification of RI distribution on 0-day-old gemma of wildtype and *Mpfrh1*^{LOF}

RI density in Tak-1, Tak-2 and *Mpfrh1*^{LOF} in % [RIs/all epidermal cells in central area] (A), occurrence and size of RI clusters in Tak-1, Tak-2, *Mpfrh1*^{LOF} and *MpRSL1*^{GOF} presented as pie charts in B and classification into wildtype-like distribution of RIs (*Mpfrh1*^{LOF11} and *Mpfrh1*^{LOF36}) and lines showing a mild cluster defect (*Mpfrh1*^{LOF34}), medium cluster defect (*Mpfrh1*^{LOF7} and *Mpfrh1*^{LOF27}) and a strong cluster defect (*MpRSL1*^{GOF}). Occurrence of RI clusters in % given in C. Tak-1 (n = counted RI cluster = 132), Tak-2 (n = 104), *Mpfrh1*^{LOF7} (n=155), *Mpfrh1*^{LOF11} (n=107), *Mpfrh1*^{LOF27} (n=174), *Mpfrh1*^{LOF34} (n=75), *Mpfrh1*^{LOF36} (n=72), *MpRSL1*^{GOF} (n = 99). Statistical significance based on student t-test is given for each line above the bars, compared to Tak-1 and Tak-2 (combined), * = p < 0.05, *** = p < 0.001, n.s. = not significant, p > 0.05

The *Mpfrh1*^{LOF} mutants fell into three classes with respect to their RI clustering phenotype – “wildtype like”, “mild” and “medium”. *Mpfrh1*^{LOF11} and *Mpfrh1*^{LOF36} showed a very similar phenotype to wildtype (Tak-1 and Tak-2) and were classified as “wildtype-like”. In *Mpfrh1*^{LOF11}, 63.6% of the RIs comprise single RIs and 23-24.3% of RIs grew in pairs. The remaining 11% were either RIs growing in clusters of 3, 4 or the maximum 5 RIs, very similar to wildtype (Tak-1 and Tak-2). Also *Mpfrh1*^{LOF36} developed very similar to wildtype (Tak-1 and Tak-2) with no obvious differences in RI clustering.

The RI clusters in *Mpfrh1*^{LOF34} are similar in size to wildtype (Tak-1 and Tak-2), but only 48% of the RIs were isolated growing RIs compared to 64% single RIs in wildtype (Tak-1 and Tak-2). Instead more RIs develop in pairs (37%) than in wildtype (25% in Tak-1 and 20% in Tak-2). RI clusters larger than 4 cells did not occur in this mutant. It was therefore classified as “mild” with respect to its RI cluster phenotype.

Mpfrh1^{LOF7} and *Mpfrh1*^{LOF27} were classified as having a “medium” RI distribution defect. In *Mpfrh1*^{LOF7} 47 % of RIs were isolated without neighbouring RIs. Similar to wildtype, 23.2 % of RI clusters constitute a group of 2 RIs and 12.3 % constitute a cluster

of 3 RIs. 17 % of the RI clusters comprise 4 or more neighbouring RIs. Among these were 6 % RI clusters that comprise 4 or 5 RIs, each. Very few clusters contained 6,8 or 9 RIs (1.3%, 0.6% and 1.9%, respectively). However clusters with so many RIs (greater than 6 RIs) were never observed in wildtype (Tak-1 or Tak-2). Also in *Mpfrh1^{LOF27}* few clusters of 7 or 8 RIs were observed (0.6 % each), a feature never observed in Tak-1 nor Tak-2 (Figure 3.7 B and C). Therefore, both *Mpfrh1^{LOF7}* and *Mpfrh1^{LOF27}* were classified as “medium”. Overall these results demonstrate that RI clusters in *Mpfrh1^{LOF}* lines are enlarged and more epidermal cells differentiate into RIs, indicating that MpFRH1 miRNA suppresses the formation of connected RIs.

Since *MpRSL1* is a direct target of MpFRH1, I hypothesise that the formation and the distribution of RI clusters in *Mpfrh1^{LOF}* is defective due to increased *MpRSL1* activity. If high levels of *MpRSL1* activity causes the development of larger RI clusters, I predict that *MpRSL1^{GOF}* lines would show similar RI clusters to *Mpfrh1^{LOF}* lines. Indeed, *MpRSL1^{GOF}* lines developed RI clusters bigger than 6 cells (3 %) and even RI clusters of more than 10 cells were observed (3 %). Because of the larger RI clusters that formed in *MpRSL1^{GOF1}* (Proust et al., 2016, first described as ST53-1, see supplementary Figure S3.3), this line was classified as “strong” regarding its RI pattern phenotype (Figure 3.7 B and C). These findings suggest that elevated *MpRSL1* expression is sufficient to promote the differentiation of RIs and that elevated *MpRSL1* expression can affect RI patterning.

Taken together these results suggest that MpFRH1 mediates suppression of *MpRSL1* in epidermal cells [adjacent to differentiated RIs] to restrict the number of adjacent RIs.

3.3.8 The shape of RI clusters is defective in *Mpfrh1*^{LOF}

Not only the size of RI clusters is defective in *Mpfrh1*^{LOF} mutants, but the formation of these clusters is also altered. While RI clusters are arranged in rows in wildtype (in Tak-1 and Tak-2), this pattern is more irregular in *Mpfrh1*^{LOF}. I quantified the number of directly neighbouring RIs for each RI in wildtype as well as in *Mpfrh1*^{LOF} and *MpRSL1*^{GOF} lines, to test if indeed pattern formation of those rhizoid cells changed.

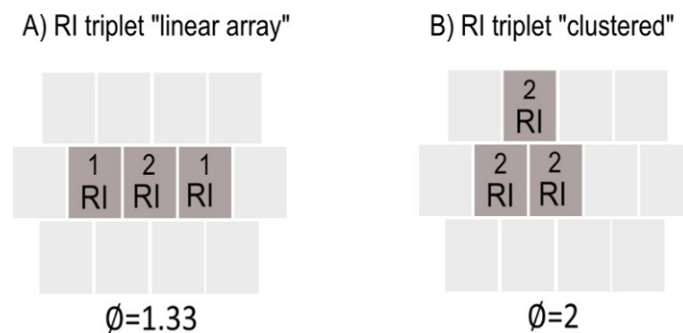


Figure 3. 8 Quantification of clustering defects

Epidermal surface (squared cells) with RIs (dark grey cells). Number indicate the count of neighbouring RIs for each RI. In A) a linear triplet typical for wildtype is shown. Outer RIs have 1 neighbouring RI, while inner RI has two neighbouring RIs. In a clustered triplet all RIs can have 2 neighbouring RIs (B). Number below each field of cells indicate the average number of neighbouring RIs for each demonstrated cluster.

Wildtype RI clusters tend to be linear, where the RIs are arranged in a linear array within the cluster. Consequently, in Tak-1 and Tak-2, a wildtype cluster of three RIs (a RI-triplet), there are always two outer RIs (with one adjacent RI) and one central RI (with two adjacent RIs), which means on average an RI in a triplet has 1.33 neighbouring RIs (Figure 3.8A). Also in larger clusters, e.g. clusters comprising four or five RIs, there are always two outer RIs and two or three inner RIs, respectively. In this arrangement there are on average 1.5 or 1.6 neighbouring RIs per RI, respectively. In *Mpfrh1*^{LOF} lines RIs in clusters of three cells can be connected to each other, i.e., they form next to each other.

Therefore, in a three-cell cluster each RI can develop 2 neighbouring RIs, which increases the average number of neighbouring RIs in the cluster (Figure 3.8B). This average number of neighbouring RIs reflects how closely clustered RIs are within a single cluster; higher values indicate clusters with many RIs abutting each other, lower values indicate that RIs in the cluster have fewer RI neighbours (as found in the linear arrays of wildtype, Figure 3.8A). In all five *Mpfrh1*^{LOF} mutants, the number of neighbouring cells in a three RIs cluster was greater than 1.33, which is the average number of neighbouring RIs in a wildtype three RIs-cluster. For example, three RIs cluster of *Mpfrh1*^{LOF7} and *Mpfrh1*^{LOF27} have approximately 1.4 (SD = 0.21 and 0.26, respectively) RI neighbours. *Mpfrh1*^{LOF11} and *Mpfrh1*^{LOF34} have 1.56 (SD = 0.34) and *Mpfrh1*^{LOF36} 1.6 (SD = 0.37) neighbouring RIs. The same trend can be seen in four RIs cluster (RI-quadruplets). In *Mpfrh1*^{LOF7}, *Mpfrh1*^{LOF11} and *Mpfrh1*^{LOF34} mutants, RI clusters containing four RIs developed approximately 1.7 (SD = 0.37, 0.45 and 0.29, respectively) neighbouring RIs, compared to exclusively 1.5 (SD = 0) RI neighbours in a four-cell RI cluster in wildtype (Tak-1 and Tak-2). Clusters of five RIs in *Mpfrh1*^{LOF27} developed 2.2 (SD = 0.28) neighbouring RIs compared to 1.6 as observed in wildtype (Figure 3.9).

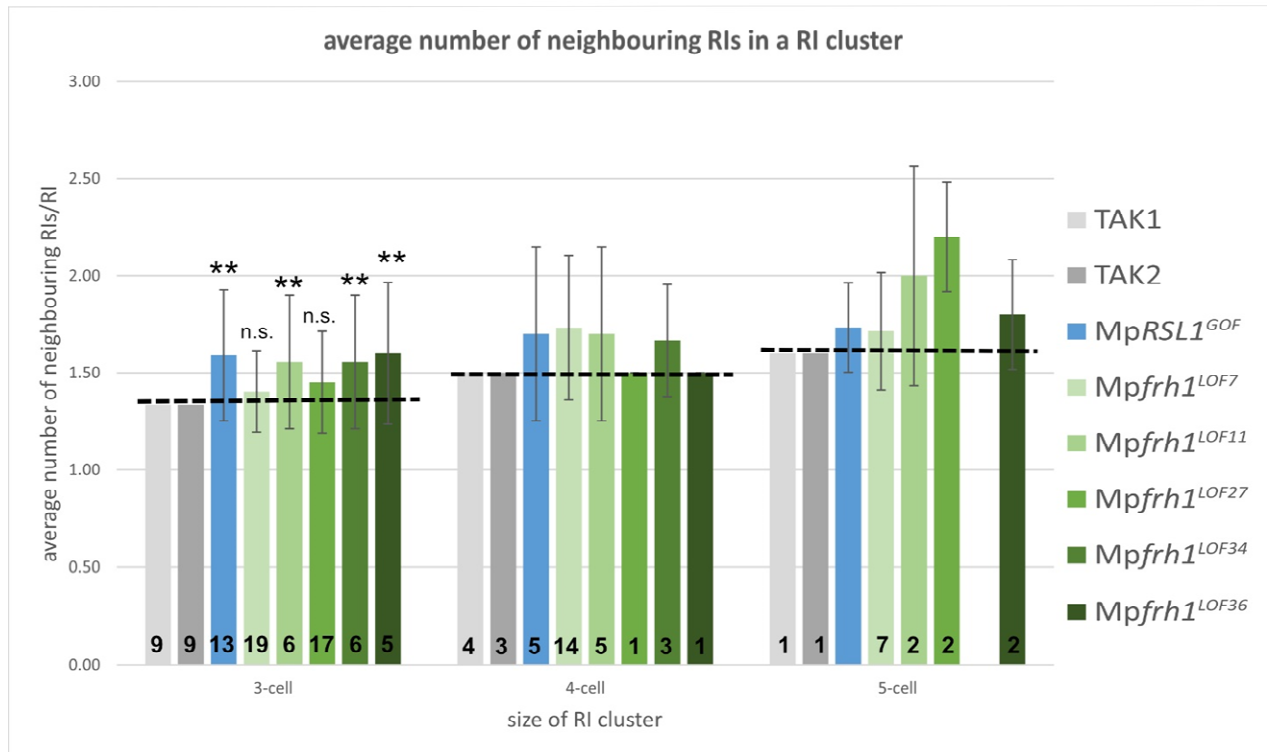


Figure 3. 9 Average number of neighbouring RIs in wildtype, *Mpfrh1*^{LOF} and *MpRSL1*^{GOF}

Mean number of neighbouring RIs growing in a 3-cell, 4-cell and 5-cell cluster in wildtype (Tak-1 and Tak-2, grey), *MpRSL1*^{GOF} (blue) and *Mpfrh1*^{LOF} (green). Numbers at the base of each bar represent number of analysed clusters. Statistical significance based on student t-test is given for each line compared to Tak-1 and Tak-2 (combined) for 3-cell cluster, ** = $p < 0.01$, n.s. = not significant, $p > 0.05$

In summary, RIs in wildtype are largely surrounded by non RIs. In RI clusters made of three or more cells, the inner RIs usually develop 2 adjacent neighbouring RIs. RIs develop more RI neighbours in *Mpfrh1*^{LOF} mutants and consequently fewer non RI neighbours. These findings support the hypothesis that *MpFRH1* suppresses the formation of RIs next to already existing RIs.

If this suppression is due to the repression of *MpRSL1* during rhizoid formation, I would expect similar RI clustering defects in *MpRSL1*^{GOF} mutants. Therefore I determined the RI clustering phenotype of *MpRSL1*^{GOF} mutants. RI clustering is higher in *MpRSL1*^{GOF}

than in wildtype. RIs of three-RIs clusters developed on average 1.59 (SD = 0.34) neighbouring RIs compared to 1.33 in wildtype. RIs in four-cell clusters developed 1.7 (SD = 0.45) neighbours and RIs in five-RI clusters developed an average of 1.73 (SD = 0.23) neighbours (compared to 1.5 and 1.6 in wildtype, respectively, Figure 3.9 + Figure S3.3). Together these results indicate that MpFRH1 suppresses the development of RI clusters by repressing the development of RIs next to each other. This suppression is likely mediated through the repression of MpRSL1 activity in epidermal cells adjacent to forming RIs.

3.3.9 MpFRH1 represses the development of mucilage papillae in adjacent cells

Mucilage papillae (MPs) are specialised multicellular structures that develop from single epidermal cells that develop close to the meristem in the apical notch of gemmae and thalli. MpFRH1 and MpRSL1 regulate the development of RIs and MPs (Honkanen et al., 2018); MPs do not develop in lines that overexpress MpFRH1 and in *Mprsl1* loss-of-function mutants. Furthermore, the promoter of *MpFRH1* is active in MPs. I investigated the formation of MPs in *Mpfrh1*^{LOF} mutants, to test the hypothesis that the patterning of MPs is impaired similarly to the patterning of RIs.

I observed phenotypic variation in the phenotype of MPs in 0-day-old gemmae of wildtype and *Mpfrh1*^{LOF} mutants. Wildtype (Tak-1 and Tak-2) gemmae usually develop characteristic oval shape with two apical notches, each harbouring one or two MPs (Figure 3.3, 3.10, 3.11). Apical notches of *Mpfrh1*^{LOF} frequently developed more MPs than those of wildtype plants. In *Mpfrh1*^{LOF7}, *Mpfrh1*^{LOF27}, *Mpfrh1*^{LOF36}, I observed up to 5 neighbouring MPs (see Figure 3.10). *Mpfrh1*^{LOF11} usually developed two MPs, but occasionally three. In *Mpfrh1*^{LOF34} the number of MPs was the same as wildtype, but MP shape was defective (see Figure 3.10). These results indicate that MpFRH1 miRNA limits

the formation of the number of MPs in apical notches of gemmae. These observations are consistent with a role for MpFRH1 in suppressing the differentiation of RIs in adjacent cells. Together these findings suggest that MpFRH1 reduces the expansion of specialised structures that originate from individual epidermal cells like RIs and MPs.

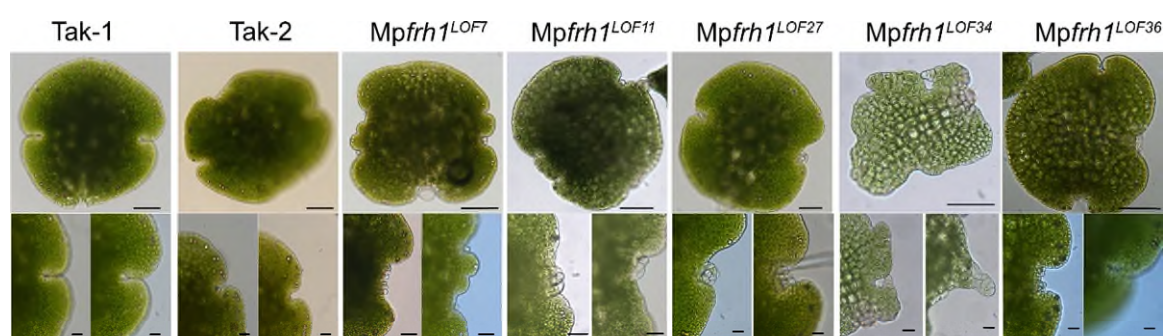


Figure 3.10 Overview of gemmae and meristematic notches harbouring mucilage papillae cells of *Mpfrh1*^{LOF} compared to Tak-1 and Tak-2

Upper row: overview image of gemmae and in lower row close up images of apical notches of *Mpfrh1*^{LOF} and Tak-1 and Tak-2. Scale bar upper row: 100µm, lower row: 25 µm

3.3.10 MpFRH1 represses the formation of meristematic notches

I also observed the presence of more meristematic notches in *Mpfrh1*^{LOF} lines than in wildtype plants. Wildtype gemmae generally developed two apical notches, 92% of the Tak-1 gemmae developed 2 apical notches and rarely (8%) developed 3 apical notches (Figure 3.11). Tak-2 gemmae developed 2 (79%) or 3 (21%) apical notches. 52% of the gemmae developed 2 apical notches in *Mpfrh1*^{LOF7} mutants. 38% of the *Mpfrh1*^{LOF7} mutant gemmae developed 3 apical notches and 10% of the *Mpfrh1*^{LOF7} mutant gemmae developed 4 or even 5 meristematic notches (5% each). Most of the gemmae of *Mpfrh1*^{LOF11} and *Mpfrh1*^{LOF27} developed 2 or 3 apical notches, but some gemmae (8% in *Mpfrh1*^{LOF11}, 10% in *Mpfrh1*^{LOF27}) formed 4 apical notches. The largest increase in the number of apical notches on gemmae was observed in the *Mpfrh1*^{LOF34} mutant. Here, 27% of gemmae

developed 4 apical notches, 36% developed 5 apical notches and 9% developed 6 apical notches. No increase in the number of apical notches was observed in the *Mpfrh1^{LOF36}* line (Figure 3.11). These results indicate that the absence of MpFRH1 miRNA results in the formation of ectopic apical notches at the margin of gemmae. This indicates that MpFRH1 suppresses the development of supernumerary apical notches. Moreover, it suggests that MpFRH1 controls putative regulators of apical notch, and by extension, meristem development.

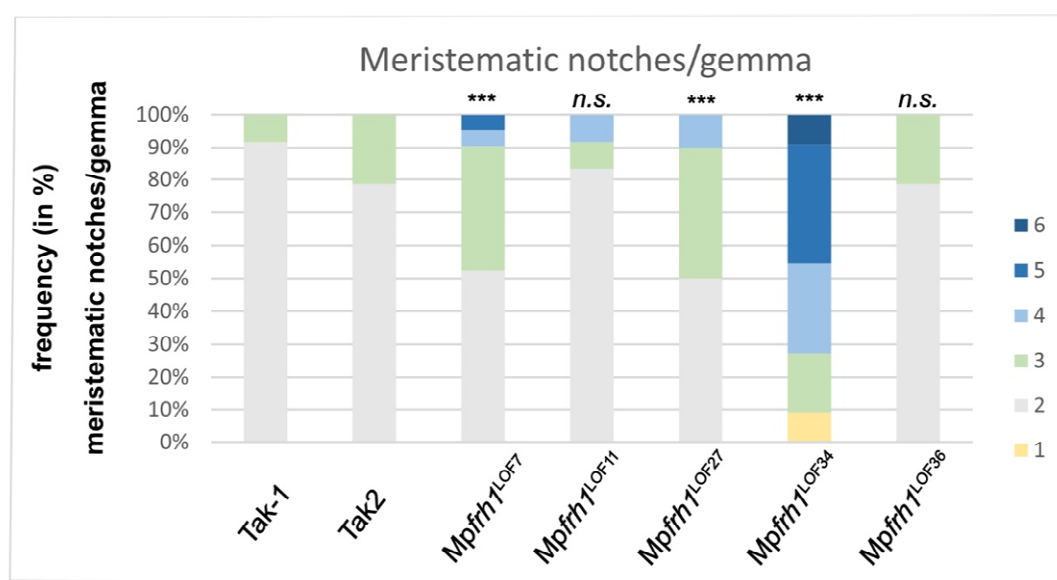


Figure 3. 11 Frequency (in %) of number of meristematic notches in 0-day-old gemmae of *Mpfrh1^{LOF}* compared to Tak-1 and Tak-2

Individual genotypes are given at the x-axis, frequency of number of meristematic notches given in %. Number of notches (1 to 6) per gemma given in colours (yellow = 1, grey = 2, green = 3, light blue = 3, blue = 5, dark blue = 6). Tak1 (n=12), Tak-2 (n=14), *Mpfrh1^{LOF7}* (n=21), *Mpfrh1^{LOF11}* (n=12), *Mpfrh1^{LOF27}* (n=10), *Mpfrh1^{LOF34}* (n=11), *Mpfrh1^{LOF36}* (n=14). Statistical significance based on student t-test compared to Tak-1 and Tak-2 (combined) : *** = $p < 0.001$, n.s. = not significant, $p > 0.05$

3.3.11 Loss of MpFRH1 function causes the development of twisted thalli

Gemmae of *Mpfrh1*^{LOF} are generally smaller and can form more than two apical notches as observed in wildtype. To observe if those apical notches are active and affect thallus development, I observed the development of gemmalings growing for 23 days.

During thallus development from gemmae, Tak-1 and Tak-2 develop a characteristic bifurcation pattern (Solly et al., 2017). The lobes surrounding a meristem expand and elongate before initiating a new apical notch at their apical end. The cycle initiates again and lobes expand and elongate before a further meristem develops on the distal region of each. In *Mpfrh1*^{LOF} this bifurcation pattern was defective. *Mpfrh1*^{LOF} gemmae were smaller than wildtype and formed more than two apical notches during gemma development, resulting in the formation of more thallus lobes than in wildtype. Images of thallus development from gemma were captured over a time course of 23 days (Figure 3.3 and in more detail in Supplementary Figure 3.2). It is clear in *Mpfrh1*^{LOF7} and *Mpfrh1*^{LOF34} that more apical notches were present and more lobes were expanding than in wildtype after 5 days. Different apices grew at different speed in *Mpfrh1*^{LOF7} and *Mpfrh1*^{LOF34} and the fast-growing lobes overgrew slow-growing lobes. This is consistent with the control of apical dominance in *M. polymorpha*, where one lobe growth greater compared with its neighbouring lobe (Davidonis and Munroe, 1972). This is especially clear after 10 days of gemmaling development. Furthermore, the regular bifurcating thallus structure of wildtype (Tak-1 and Tak-2) was defective in *Mpfrh1*^{LOF} mutants. After 23 days there were a variety of thallus defects. In *Mpfrh1*^{LOF7} and *Mpfrh1*^{LOF11} the expansion (elongation and bifurcation) of the thallus was defective and thallus lobes were curled, preventing the thallus from growing flat along the surface of the growth medium. Instead, the mutant thalli developed a crinkled phenotype. Thallus curling was less severe in *Mpfrh1*^{LOF27}, *Mpfrh1*^{LOF34} and *Mpfrh1*^{LOF36} mutants. However, the apical notches were

located closer to each other than in wildtype causing the thalli to grow on top of each other, giving the thallus a twisted phenotype.

The developmental defects that can be observed in young gemmae and developing thallus of *Mpfrh1*^{LOF} mutants; smaller gemmae size, ectopic apical notches and thallus development suggest a role for the MpFRH1 miRNA in the repression of meristem initiation, the control of thallus development as well as the spatial regulation of the pattern of MPs and RIs.

3.3.12 Small INDELs are sufficient to induce MpFrh1⁻ phenotype

MpFRH1 is a regulatory miRNA defining the development of RIs, MPs, apical notches and thallus growth. Those conclusions were made based on the defective phenotype of *Mpfrh1*^{LOF}. Those loss of function lines harbour a deletion within the MpFRH1 gene of up to 600 bp. To confirm that the observed defects are indeed caused by the loss of the 21 bp long miRNA MpFRH1 and no other regulatory sequences within the MpFRH1 sequence, I generated small INDELs (INsertions/DEletions) using the CRIPR/Cas9 system with one sgRNA targeting the 5' of the miRNA MpFRH1 sequence. After sequencing approximately 50 transformants, I selected two lines with a verified mutation within the MpFRH1 gene. *Mpfrh1*^{INDEL14} has a deletion of 8 bp around the mature MpFRH1 sequence (Figure 3.12 A and Supplement data 3.6.1). One of the bases is part of the mature miRNA sequence while the other 7 bp are part of the putative hairpin structure. *Mpfrh1*^{INDEL34} has a deletion of 4 bp, which do not correspond to the mature miRNA sequence, but are part of the hairpin structure (Figure 3.12 A and Supplement data 3.6.1). I was predicting the secondary RNA structures of those mutants using the 'RNAfold webserver' (Figure 3.12 B). Those small INDELs are likely to affect the secondary RNA structure, which is essential for miRNA processing, therefore MpFRH1 processing and function is likely to be affected in those lines.

To test if these mutations are sufficient to cause similar defective phenotypes as *Mpfrh1*^{LOF} mutants caused by larger deletions, I investigated if *Mpfrh1*^{INDEL14} and *Mpfrh1*^{INDEL34} also develop supernumerary MPs, as this is a very characteristic feature of those loss of function lines. *Mpfrh1*^{INDEL14} and *Mpfrh1*^{INDEL34} mutants developed supernumerary MPs (Figure 3.12 C). The development of the same phenotype in small mutations in the DNA sequence encoding the MpFRH1 miRNA as observed in the larger

deletions (in section 3.3.9) verifies that the defects are due to loss of MpFRH1 miRNA function.

To support the hypothesis that the 21 bp MpFRH1 miRNA is causing the described phenotypic defects and no other regulatory genes that were deleted in *Mpfrh1*^{LOF7/11/27/34/36} mutants, I investigated the MpFRH1 allele again. Based on transcriptomics there are no other transcripts annotated within the deleted DNA sequence (Bowman et al., 2017). Furthermore, no other miRNA was reported to be encoded within the MpFRH1 allele (Tsuzuki et al., 2016). Those results are consistent with the hypothesis that the phenotypes that we observe in *Mpfrh1*^{LOF} lines with deletions up to 600 bp are caused by the lack of the 21 bp long MpFRH1 miRNA.

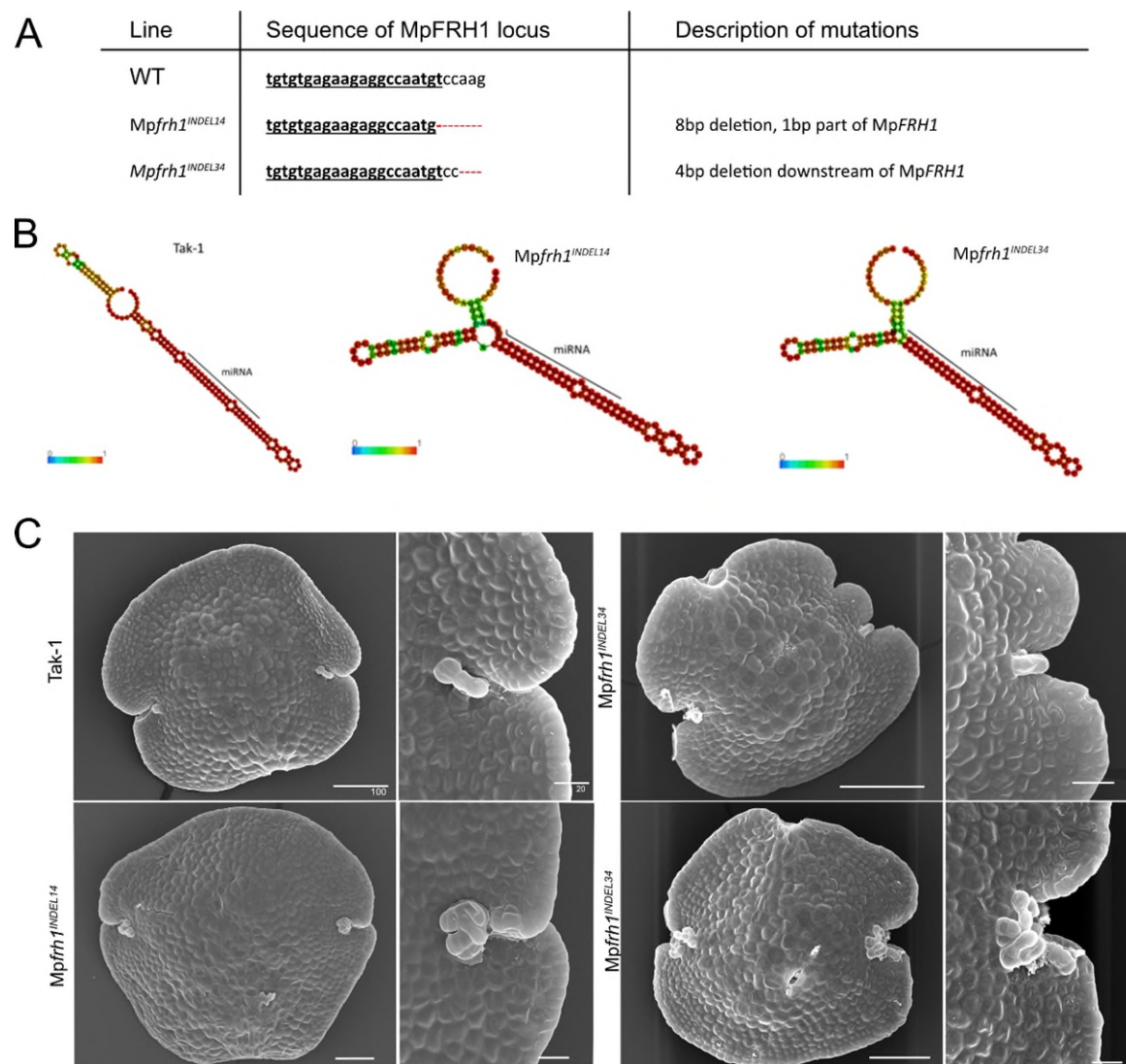


Figure 3. 12 Mutation at MpFRH1 allele in *Mpfrh1*^{INDEL14/34} and their predicted secondary structure of MpFRH1 transcript as well as their developmental defect of mucilage papillae cells on 0-day-old gemmae

A) Sequence of MpFRH1 allele of Tak-1 and *Mpfrh1*^{INDEL14} and MpFRH1^{INDEL34}

B) Secondary structure prediction of MpFRH1 transcript in Tak-1, *Mpfrh1*^{INDEL14} and *Mpfrh1*^{INDEL34} and C) SEM image of Tak-1 and *Mpfrh1*^{INDEL14} and *Mpfrh1*^{INDEL34} gemma

showing ectopic mucilage papillae cells as well as supernumerary apical notches

3.4 Discussion

Here, I showed that the miRNA MpFRH1 controls the spatial distribution of specialised epidermal structures like unicellular RIs and multicellular MPs. RIs are evenly distributed on the epidermal surface and usually develop as individual cells or grow in small clusters of 2, 3 or 4 cells. Those clusters are enlarged in *Mpfrh1*^{LOF} with RIs growing in clusters up to 9 cells. A similar change is observed in respect to MPs. Usually one or two papillae grow on one side of the thallus around the apical cell, whereas several papillae grow in *Mpfrh1*^{LOF}. In total I generated and characterised 5 independent *Mpfrh1*^{LOF} lines using the CRISPR/Cas9 system, with various strength of the *MpFrh1*- phenotype. During T-DNA mutagenesis the T-DNA harbouring the expression sequence for the Cas9 endonuclease and the sgRNA(s) randomly inserts into different loci of the genomic DNA, generating lines with distinct genetic backgrounds. Those various insertions sites can influence the expression of affected and surrounding genes, which can have various effects on the expression of the *Frh1*- phenotype. To limit the genetic background variation, the *Mpfrh1*^{LOF} lines generated should be backcrossed to parental wildtype accessions Tak-1 or Tak-2. Nevertheless, enlarged rhizoid clusters were observed in all *Mpfrh1*^{LOF} lines, indicating that indeed the lack of MpFRH1 activity causes the formation of clustered rhizoid cells. Since similar affects were observed regarding the formation of MPs, these findings indicate that MpFRH1 generally represses the formation of specialised structures originating from an epidermal cell like RIs and MPs to ensure a restricted pattern of those structures.

MpFRH1 binds directly to *MpRSL1* mRNA to cause post transcriptional silencing. *MpRSL1* has been shown to be essential for RI and MP development (Proust et al., 2016) and here I reported that RI clusters also develop in *MpRSL1*^{GOF}. Hence, I conclude that MpFRH1 - a suppressor of structures derived from single epidermal cells - is necessary to

repress MpRSL1 expression - an activator of specialised epidermal structures. The reduction of MpRSL1 expression ensures the development of individual epidermal cells into specialised epidermal structures and thereby establishes epidermal patterning.

The interaction of activating and repressing regulators is a common mechanism in development. It has previously been described in the development of trichomes on the leaf epidermis of *A. thaliana*. Arabidopsis trichomes are unicellular specialised epidermal cells which are uniformly distributed. Coordinated expression of positive and negative regulators specifies the trichome cell fate early in epidermal development (Scheres, 2002; Pesch and Hülskamp, 2004; Ishida et al., 2008). Throughout the protoderm, both positive and negative regulators are expressed evenly. During leaf development their expression becomes more restricted to initiating trichome cells (Pesch and Hülskamp, 2009). Concretely, trichome patterning involves the positive regulators R2R3-Myb TF GLABRA1 (GL1) and WD-40 scaffold protein TRANSPARENT TESTA GLABRA1 (TTG) to form a transcriptional complex with the bHLH TF GLABRA3 (GL3) and ENHANCER OF GL3 (EGL3) (Larkin, 1994; Payne et al., 2000; Bernhardt et al., 2003; Zhao et al., 2008). This complex then activates the expression of the homeodomain TF GLABRA2 (GL2), which promotes trichome cell differentiation (Rerie et al., 1994; Masucci et al., 1996). This complex also directly induces transcription by binding to the promoter region of the inhibitors, which are in total six single repeat Myb proteins that act redundantly e.g. CAPRICE (CPC), TRIPTYCHON (TRY), ENHANCER OF TRY (ETC1), ETC2, ETC3 and TRICHOME-LESS1 (TCL1). Once transcribed and translated, these inhibitor proteins move to the neighbouring cells via plasmodesmata, where they can counteract the activator complex by competing with GL1 (Schellmann et al., 2002). TTG/GL3/EGL3 bound to a CPC/TRY protein supresses the activation of GL2 transcription and hence trichome formation (Ishida et al., 2008; Torii, 2012).

Combined, their regulatory relations appear to satisfy the basic circuitry of a well described mathematical model named after Alan Turing. This Turing-model describes how the interaction of an activator and an inhibitor is sufficient to generate distributed individual peaks of the activator. Several criteria must be fulfilled for a Turing model. Activator and inhibitor are initially evenly expressed. Stochastic changes of their distribution initiate an increase in activator abundance due to its self-activating property. An increase of activator expression also mediates the activation of the expression of the inhibitor. The inhibitor diffuses more quickly to the neighbouring cells than the activator (if applicable). This causes a lateral inhibition around cells with high activity of activator and hence of those cells that will differentiate.

Even though evidence suggest that trichome pattern formation may conform to a Turing model, there are data that are inconsistent with a Turing model operating during trichome patterning in *A. thaliana*. First, self-activation of a positive regulator, GL1 or GL3 has not been shown (Morohashi and Grotewold, 2009; Torii, 2012). Second, *Atttg* mutants exhibit opposing effects as positive or negative regulator of trichome patterning. While *Atttg* null mutants produce no trichomes, weak allele lines develop trichome clusters (Larkin et al., 1999, Torii, 2012). Hence, while trichome formation in Arabidopsis is one of the best studied systems of patterning formation, and the basic circuitry resembles the activator-inhibitor model of Turing, those data suggest additional mechanism operating during trichome formation.

Interestingly, the loss of activators (e.g. GL1, GL2) or increased activity of suppressors (e.g. CPC, TRY, ETCs) during trichome development, result in lack of trichomes while the loss of inhibitor or constitutive activation of the activator causes leaves with excessive, clustered trichomes (Zhang, 2003; Wester et al., 2009; Torii, 2012). These observations are similar to our observations about MpRSL1 and MpFRH1 during rhizoid

formation, where the loss of MpRSL1 or the increased activity of MpFRH1 result in no rhizoid formation and the loss of MpFRH1 activity result in the formation of RI clusters.

I hypothesise that Mp*RSL1* and Mp*FRH1* are two regulatory genes – an activator and an inhibitor, respectively – controlling rhizoid formation on *M. polymorpha* thalli, due to a similar mechanism than trichome formation on Arabidopsis leaves. In a simplified way trichome formation occurs when the positive regulator complex operates (e.g. AtGL1 bound to AtGL3/AtEGL3/AtTTG1) by activating AtGL2. At the same time, it activates its negative regulator (e.g. TRY or its redundant acting proteins CPC, ETC2, ETC3 and TCL1), which moves to neighbouring epidermal cells. There, it binds to the AtGL3/AtEGL3/AtTTG1 complex and in this way suppresses the positive regulator complex from activating AtGL2. Thereby, TRY represses trichome differentiation in neighbouring cells through lateral inhibition.

If MpRSL1 and MpFRH1 operate in a similar way, I would predict that MpRSL1 operates in epidermal cells undergoing rhizoid formation. At the same time MpRSL1 activates MpFRH1, which acts as a mobile signal moving to neighbouring epidermal cells to suppress MpRSL1 expression and hence rhizoid formation in neighbouring cells. While we showed that MpRSL1 positively regulates Mp*FRH1* transcription and MpFRH1 represses Mp*RSL1* mRNA accumulation (Honkanen et al., 2018), nothing is known about the ability of MpFRH1 to act as a mobile signal. Within the next chapter I am investigating the spatial distribution of Mp*RSL1* mRNA as well as the activity of MpFRH1 in mature gemmae to investigate the mechanism of Mp*RSL1*-MpFRH1 interaction during rhizoid formation.

3.5 Material and Methods

3.5.1 Plant material and growth conditions

M. polymorpha accessions Tagaragaike-1 (Tak-1, male) and Tagaragaike-2 (Tak-2, female) were used as wildtype (Ishizaki et al., 2008). Plants were grown on sterile plates containing Johnson's medium. Plants were growing under continuous white light illumination ($56 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) at 23 °C.

For RNA extraction plants were grown on Johnsons medium covered with a disc of cellophane membrane, to make harvesting easier and avoid agar contaminating sampling.

To measure rhizoid growth 2-day-old gemmalings were transferred into individual sterile conical tubes (Sarstedt, 60ml, 60.596.001) filled with 50ml Johnson mediums supplied with 0.8% phytigel (Sigma-Aldrich) instead of agar. To guarantee oxygen flow the lids were perforated and sealed with sun cap closures (Sigma Aldrich, S5939-500EA).

To initiate gametophore development to further carry out crossing, plants were grown in a growth chamber at 23 °C under 16 hours light:8 hours dark photoperiod on 1:3 mixture of vermiculite and John Innes No. 2 compost. Sexual reproduction was induced by far-red light irradiation as described in Chiyoda et al., 2008

3.5.2 Design of sgRNAs and plasmid generation to generate *Mpfrh1* loss of function alleles

The generation of CRISPR/Cas9- mediated mutations followed the protocol published by Sugano et al. (2014). The gRNAs were designed by searching for a PAM sequence (NGG), and protospacer (20nt sequence downstream of PAM) which is unique to the *MpFRH1* allele. For generating individual mutations at the *MpFRH1* miRNA sequence, the

protospacer 'agaagaggccaatgtccaag' was inserted into the gRNA expression vector, *pro*MpU6-1:gRNA or MpEN03, through BsaI overhangs. MpEN03 was recombined with the Cas9 expression vector, MpGE010 through LR recombination.

For using two gRNA at the same time, the gRNA expression vector MpEN03 was modified by Holger Breuninger, by integrating a second *pro*MpU6-1:gRNA transgene, generating pHB453 (Figure S3.4). pHB453 was digested using BbsI (for insertion of the first protospacer) and after successful insertion of first protospacer digested with BpiI to insert second protospacer. The protospacers were inserted into digested pHB453 through BbsI or BpiI overhangs, respectively. pHB453 was recombined with the Cas9 expression vector, MpGE010 through LR recombination, and verified through sequencing the scaffolds harbouring the protospacer sequences. Following successful cloning of expression vector, it was transformed into *Agrobacterium tumefaciens*.

3.5.3 *Agrobacterium* mediated transformation of *M. polymorpha* sporelings

Sporelings of *M. polymorpha* were transformed following the protocol from Ishizaki et al. (2008), which is following summarized. Sporangia of a cross between Tak-1 and Tak-2 were dried in a silica drying beads (Fisher Scientific, 10611573) containing basket for at least 10 days, before frozen at -70°C upon usage. 1-2 sporangia per transformation were surface sterilised using 0.1% sodium dichloroisocyanurate (NaDCC, Sigma) and let grow for 7 days in 25ml M51C medium in 125ml vented tissue culture flasks under continues white light conditions (light intensity of 38 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at 23 °C. A single colony of *Agrobacterium tumefaciens* strain GV3101 carrying the vector of interest was inoculated into 5 ml LB medium containing 25 $\mu\text{g}/\text{mL}$ gentamycin, 100 $\mu\text{g}/\text{mL}$ rifampicin, and 50 $\mu\text{g}/\text{mL}$ spectinomycin, and cultured with shaking at 28 °C for 2 days. The pellet of 2ml

saturated culture (generated by centrifugation at 2000 x g for 15 minutes) was resuspended in 10 ml M51C liquid medium containing 100 μ M acetosyringone (Sigma AldRIh, 97%), and cultured for a further 3- 6 h with shaking at 28 °C. The 7-day-old cultures of *M. polymorpha* sporelings were then supplemented with 100 μ M acetosyringone, and 1 mL of the acetosyringone induced bacterial culture. The sporelings and bacteria were co-cultivated with shaking at 120 rpm under continuous white light (56 μ mol photons $\text{m}^{-2}\text{s}^{-1}$) at 18°C for 2 days. Sporelings were then collected on a sterile 40 μ m cell strainer (Fisher Scientific) and rinsed thoroughly with sterile distilled water. The sporelings were spread on selection plates of Johnson's agar with 100 μ g/mL cefotaxime to inhibit *A. tumefaciens* growth and 10 μ g/mL hygromycin to select for transformed plants. After 14 days transformants were isolated for further growth and selection. Transformants were verified by sequencing the genetic region of interest.

3.5.4 DNA extraction and genotyping

DNA of individual plants was extracted following an adapted protocol from Porebeski et al. 1997. About 1x1cm piece of plant tissue was harvested into 1.5 ml Eppendorf tubes in liquid N² and ground into fine powder (either using a pestle or a tissue grinder). 500 μ l were added into each tube and incubated for 5-15 minutes at room temperature. 250ul Phenol: chloroform: isoamylalcohol (25: 24: 1) were added, well mixed and centrifuged at 13000rpm for 1 minute. The upper phase was transferred into a new Eppendorf tube with 500ul isopropanol and spun for 10 minutes at 13000 x g. The supernatant was discarded and the DNA pellet was washed twice by adding 500ul 70% ethanol, followed by centrifugation at 13000 x g for 5 minutes. The ethanol was removed to let the pellet dry. DNA concentration was determined using a spectrophotometer.

Transformants were confirmed by amplifying the genomic region of interest using Phusion High-Fidelity DNA Polymerase (ThermoFisher) following manufacturer's instruction. To confirm deletions of *Mpfrh1*^{LOF7,11,27,34 and 36} primer were designed about 200 bp upstream and downstream of the first and second sgRNA target site, respectively. The PCR product was run on a 1% agarose gel and the PCR fragment of interest gel purified using the GeneJET Gel Extraction Kit (ThermoFisher) following manufacturer instructions. The PCR amplicon was sequenced using SangerSequencing supplied by Source Bioscience (<https://www.sourcebioscience.com>) and analysed for mutations.

3.5.5 qRT-PCR

RNA extraction, DNase treatment and cDNA synthesis followed the protocol from chapter 2.4.1 – 2.4.2. The same primer pairs than above were used to amplify *MpAPT* and *MpRSL1* following the protocol from 2.4.3.

3.5.6 Imaging

23-day-old thalli were imaged using a NIKON D750 camera (John Baker) with a 25mm focal length

Light microscopy

Plants were imaged using a Leica DFC310 FX camera connected to a Leica M165 FC stereomicroscope. Gemmae were imaged using a compound microscope connected to an Olympus camera.

Confocal laser microscopy / PI staining

Cell walls were stained using propidium iodide (PI). Gemmae were incubated in 5ug/ml PI solution for (5-)10 minutes. PI solution was removed and gemmae washed twice with

MilliQ water. Gemmae were mounted on a slide with heated 0.2% agar solution. Once Agar solidified gemmae were imaged immediately. Images were acquired with a Leica SP5 confocal laser microscope and the Leica Application Suite (LAS) software using either a Leica HCX PL Fluotar 10x/0.30 or HC PL APO 20x/0.75 IMM CORR CS2 lense. PI and chlorophyll were excited at 543 nm and emission was collected between 561 and 640 nm for PI and between 680-700nm for chlorophyll.

Scanning Electron Microscopy

Gemmae were fixed in 100% dry methanol for 30 minutes and then washed twiced with 100% dry ethanol. Gemmae were stored in 100% dry ethanol at 4°C upon processing. The samples were dried at the critical point using a Tousimis Autosamdri-815, then mounted on aluminium stubs (Agar Scientific) using double-sided carbon adhesive discs (Agar Scientific), and coated with a mixture of gold and palladium using an SC7640 sputter coater (Quorum Technologies). The samples were imaged immediately with a JSM-5510 scanning electron microscope (JEOL) at an operating voltage of 15kV.

3.5.7 Image analysis

Image analyses and plant/cell measurements were performed in ImageJ.

3.5.8 Media and buffer

Johnsons medium

Component	Final concentration
KNO ₃	6 mM
MgSO ₄	1 mM
Ca(NO ₃) ₂ ·4H ₂ O	4 mM
KCl	25 µM
H ₃ BO ₄	10 µM
MnSO ₄ ·4H ₂ O	1 µM
ZnSO ₄ ·7H ₂ O	1 µM
CuSO ₄ ·5H ₂ O	250 nM
(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	250 nM
FeSO ₄ ·7H ₂ O	25 µM
Na ₂ EDTA	25 µM
NH ₄ H ₂ PO ₄ -	0.6 mM
(NH ₄) ₂ SO ₄	0.4 mM
inositol	0.55 mM
MES	0.533 g/l (w/v)
sucrose	1 % (w/v)
agar(Sigma-Aldrich)	1.4 % (w/v)

pH adjusted to 5.6 using 1M KOH

M51C medium

Component	Final concentration
KNO ₃ (Sigma)	19.8 µM
NH ₄ NO ₃ (Sigma)	5 µM
MgSO ₄ (Fisher Scientific)	1.5 µM
CaCl ₂ (Sigma)	2 µM
KH ₂ PO ₄ (Sigma)	2 µM
EDTA-Fe(III) (Sigma)	110 nM
Na ₂ MoO ₄ (Sigma)	1 µM
CuSO ₄ (Sigma)	100 nM
CoCl ₂ (Sigma)	100 nM
ZnSO ₄ (Sigma)	7 µM
MnSO ₄ (Fisher Scientific)	40 µM
H ₃ BO ₃ (Fisher Scientific)	49 µM
myo-inositol (Sigma)	555 µM
nicotinic acid (Sigma)	8 µM
pyridoxine hydrochloride (Sigma)	5 µM
thiamine hydrochloride (Sigma)	30 µM
sucrose (Sigma)	58 mM
L-glutamine (Sigma);	2 mM
casein hydrolysate (Fluka Analytical);	0.1% w/v

pH adjusted to 5.8

CTAB buffer

Component	Final concentration
Tris-HCL pH 8	100 mM
NaCl	1.4 M
EDTA pH 8	20 mM
CTAB	2 %

3.6 Supplementary Data

3.6.1 Sequence of MpFRH1 allele in Mpfrh1^{LOF}

Mpfrh1^{LOF7}

catcattcggcactctcttctgtacaaataagtacttccatcagcagacgacatcagcttgaacttgctcactcatctccccctcccc
cctccccctccccgcagccctcaacagcgccatagagtcccagacttcccactggccggccccatctgaaccttctcttctctc
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gttccggtataattccagcgctagcagttgctggagtcgtggtgatgggtgggtgtagtagtagtcatagtgtaccgcattaa
gatcctctccagtcgcttgagctgcgccacgattctgctcgtgcagcagcttggcttgggacgatttgcgtcgttcagatcgaaat
tcaacaaaagctgcagcacgaatttggattggatgagcaaccgcagataatagatagcacagccaaacagcaccgcgtgggt
ggcggccgtcactgacacagacagatagcgagctagatagatagacagatagacagaaatgttgacattctccagggtgcagc
agggaattcggccactttccaggggatcatcaccatcactcggatagactgccacgcagtttcttggctgcaagcggggcaaag
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ctgaaggatgcagattcgtagagactccgttactgatgcaccgaacttgggagctcggatcacaccctcgtgcactcaccacac
cggcaggaggattgattgagaatctagatttgtgctgcagccccgagctgtatggaattcattaccctcaataaatttgccttgcc

Mpfrh1^{LOF11}

Catcattcggcactctcttctgtacaaataagtacttccatcagcagacgacatcagcttgaacttgctcagcctcatctccccctc
ccccctccccctccccgcagccctcaacagcgccatagagtcccagacactagcgtggaattataccggaactggactaacc
aactacccaccgcccgcacgccgagccgagctagctagactgaactacggggcaggtctagggagaagccgatgagaga
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ggggaggccgccaccacgcggtgcggcgtgaagcgagtttcttggctgcaagcgggcaaagatggagattctgcacggg
ttggcgactgtagccttaggatggcgtgctccagttacaaacatactcaggatggccaggggacactgaaggatgcagattcgt
agagactccgttactgatgcaccgaacttgggagctcggatcacaccctcgtgcactcaccacaccggcaggaggattgattg
agaatctagatttgtgctgcagccccgagctgtatggaattcattaccctcaataaatttgccttgcc

Mpfrh1^{LOF27}

Catcattcggcactctcttctgtacaaataagtacttccatcagcagacgacatcagtcttgaacttgctcactcatctccccctccc
ccctccccctcccgagccctcaacagcgccatagagtcagacttcccactggccggccccatctgaacctcttctcttctct
catcggcttctccctagacctgccccgtagttcagttctagctagctcggctcggcgtggcggggcggtggggtagttggtagtcc
agttccggtataattccagcgctagcagttgctggagttgcaggggatcatcaccatcactcggatagactgccacgcagttttctt
tggctgcaagcgggcaaagatggagattctgcacgggttggcgactgtagccttaggatggcgtgctccagttacaaacatac
tcaggatggccaggggacactgaaggatgcagattcgtagagactccgttcactgatgcaccgaacttgggagctcggatcac
accctcgtgcactcaccacaccggcaggaggattgattgagaatctagatttgctgcagcccccgagctgtatggaattcatta
cccgtaataaatttgcttgcc

Mpfrh1^{LOF34}

Catcattcggcactctcttctgtacaaataagtacttccatcagcagacgacatcagtcttgaacttgctcactcatctccccctccc
ccctccccctcccgagccctcaacagcgccatagagtcagacttcccactggccggccccatctgaacctcttctcttctct
catcggcttctccctagacctgccccgtagttcagttctagctagctcggctcggcgtggcggggcggtggggtagttggtagtcc
agttccggtataattccagcgctagcagttgctggagtcaggggatcatcaccatcactcggatagactgccacgcagttttcttg
gctgcaagcgggcaaagatggagattctgcacgggttggcgactgtagccttaggatggcgtgctccagttacaaacatactc
aggatggccaggggacactgaaggatgcagattcgtagagactccgttcactgatgcaccgaacttgggagctcggatcacac
cctcgtgcactcaccacaccggcaggaggattgattgagaatctagatttgctgcagcccccgagctgtatggaattcattacc
cgtcaataaatttgcttgcc

Mpfrh1^{LOF36}

Catcattcggcactctcttctgtacaaataagtacttccatcagcagacgacatcagtcttgaacttgctcactcatctccccctccc
ccctccccctcccgagccctcaacagcgccatagagtcagacttcccactggccggccccatctgaacctcttctcttctct
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gccacgcagttttcttgctgcaagcgggcaaagatggagattctgcacgggttggcgactgtagccttaggatggcgtgctc
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ggagctcggatcacaccctcgtgcactcaccacaccggcaggaggattgattgagaatctagatttgtgctgcagccccgagc
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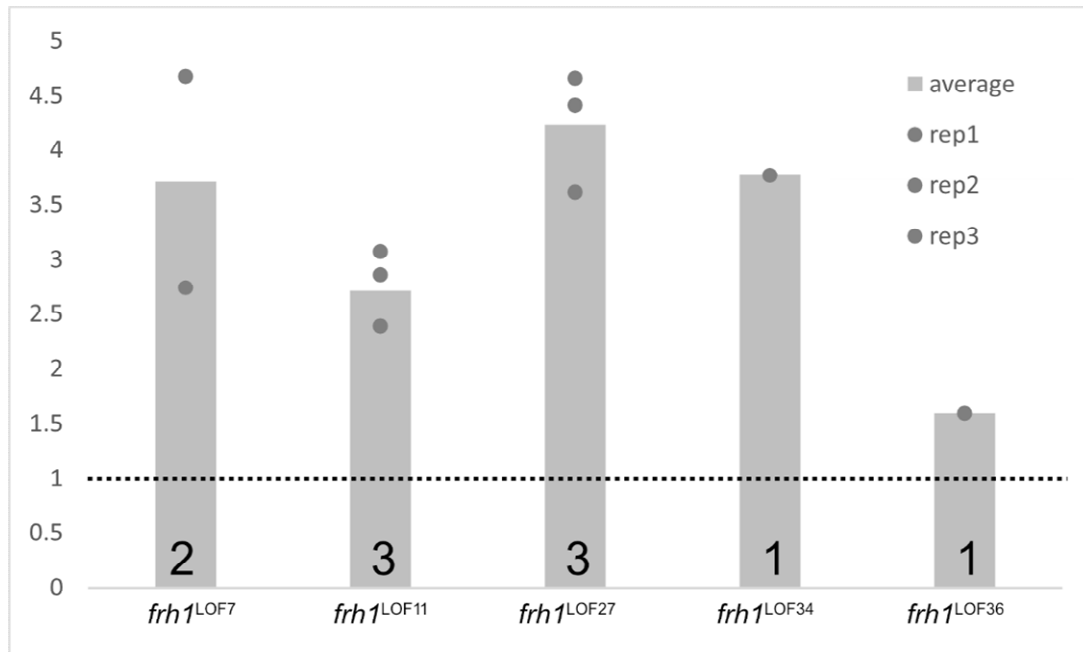
Mpfrh1^{LOF-INDEL14}

gcacattggtctcttcacgcacaaacatcgaacccttgtggcatctgtgtttgtgtgagaagaggccaatg~~ccaaagcg~~gaaaaac
cccttaaattcgaggacgattcagccgcagtcagcagcagtggtcagtcagagtaagtgggtctatgttgtgcgtgaaacagAAC
acaattttctcgtgctaacgaggtgg

Mpfrh1^{LOF-INDEL34}

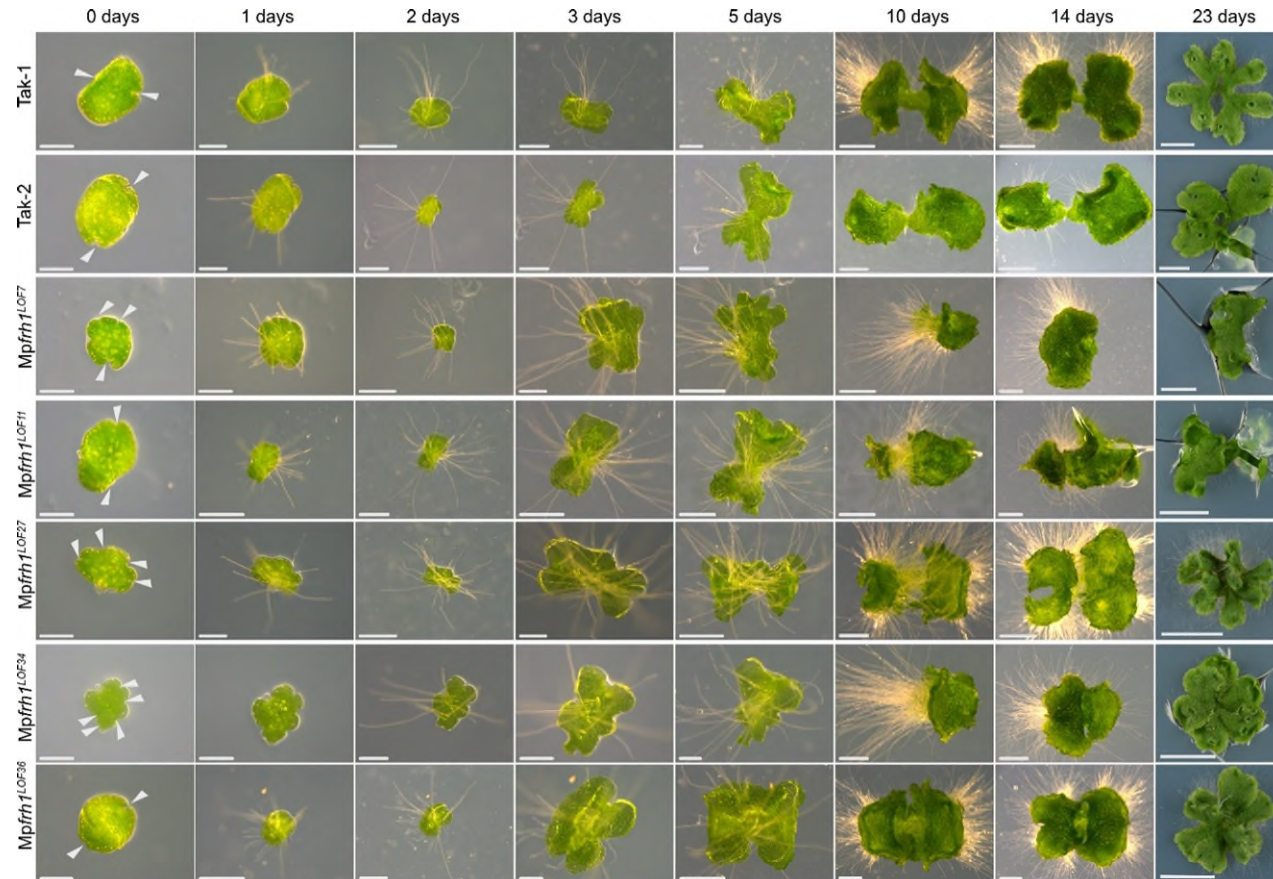
gcacattggtctcttcacgcacaaacatcgaacccttgtggcatctgtgtttgtgtgagaagaggccaatg~~ccaaagcg~~gaaaaac
cccttaaattcgaggacgattcagccgcagtcagcagcagtggtcagtcagagtaagtgggtctatgttgtgcgtgaaacagAAC
acaattttctcgtgctaacgaggtgg

3.6.2 qPCR data of *Mpfrh1*^{LOF} relative to wildtype



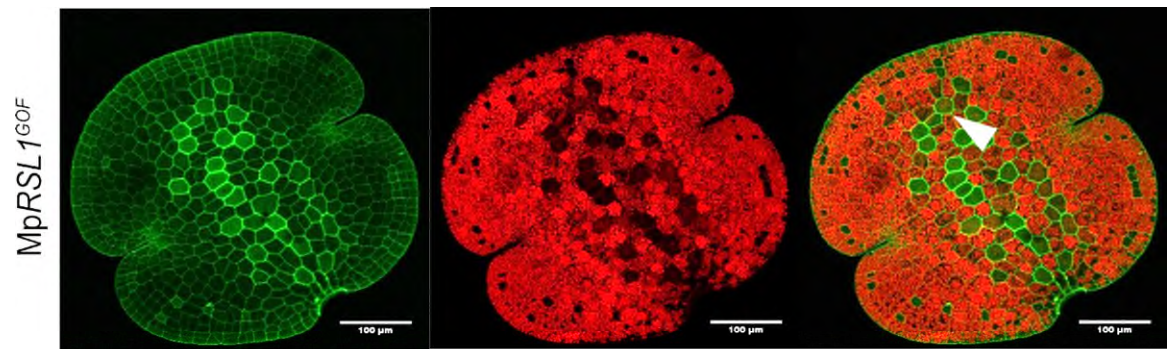
Supplementary Figure S3. 1 x-fold change of *MpRSL1* mRNA abundance in 7-day-old gemmalings of *Mpfrh1*^{LOF} compared to wildtype (Tak-1/Tak-2)

Relative *MpRSL1* mRNA abundance of 7-day-old gemmalings in *Mpfrh1*^{LOF} was compared to relative *MpRSL1* mRNA abundance in wildtype (Tak-1 and Tak-2 combined, dotted line, n=6) and the x-fold times change calculated. Number of biological replicates indicated at the bottom of each bar



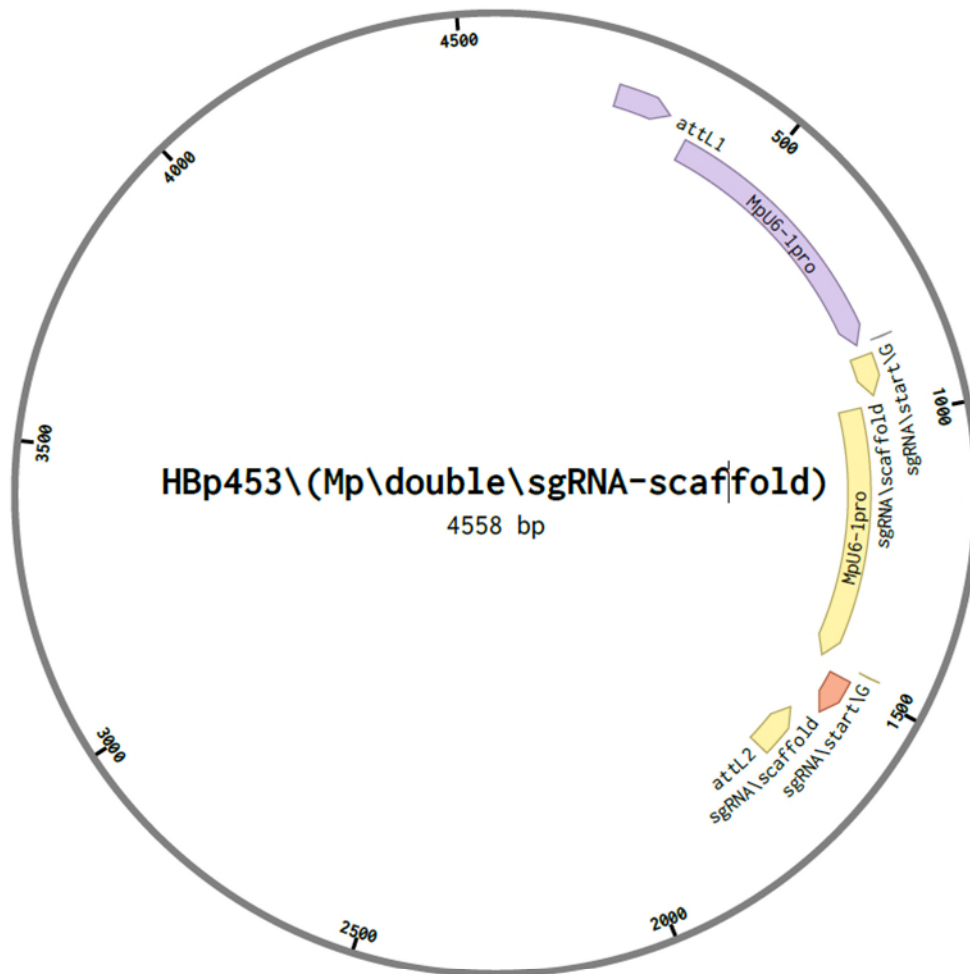
Supplementary Figure S3. 2 Gemmaling development of *Mpfrh1*^{LOF} and wildtype over 23 days

Arrowheads indicate apical notches. Scale bars: 0 days: 200 μ m; 1 day : 200 μ m (except *Mpfrh1*^{LOF11} and *Mpfrh1*^{LOF36}, here 500 μ m); 2 days: 500 μ m (except *Mpfrh1*^{LOF34}, here 200 μ m); 3 days: 200 μ m (except Tak-1, Tak-2, *Mpfrh1*^{LOF11}, here 500 μ m); 5 days : 500 μ m (except *Mpfrh1*^{LOF34}, here 200 μ m); 10 days: 1 mm (except *Mpfrh1*^{LOF27,34,36}, here 500 μ m); 14 days: 2 mm for wildtype, 1 mm for *Mpfrh1*^{LOF}, 23 days: 1 cm.



Supplementary Figure S3. 3 RI patterning in *MpRSL1^{GOF}*

RIs in *MpRSL1^{GOF}* in 0-day-old gemmae. Autofluorescence of chlorophyll colored in red, PI emission colored in green. White arrowhead highlights a RI with 4 neighbouring RIs. Scale bar = 100 μm



Supplementary Figure S3. 4 Vector map of pHB453

Vector used for CRISPR/Cas9 mutagenesis using two sgRNAs. Two pro_{MpU6} driving the expression of sgRNA1 and sgRNA, respectively.

Chapter 4

MpFRH1 and MpRSL1 act during rhizoid differentiation to control rhizoid patterning in *Marchantia polymorpha*

4.1 Abstract

The precise spatiotemporal activity of transcription factors (TFs) during development facilitates co-ordinated cell divisions and cell differentiation. However, the activity of these TFs is itself controlled by key regulators like miRNAs. MiRNAs are 21 bp short RNA molecules that bind through complementary base pairing to the mRNA of their specific target genes, mainly TFs. The miRNA-mRNA interaction mediates post transcriptional silencing of target genes by either cleaving the mRNA or blocking the translational machinery. MiRNAs can therefore function to reduce the activity of TFs to defined cell types to a very specific time point in development.

In *M. polymorpha* the miRNA MpFRH1 negatively regulates the expression of the bHLH TF MpRSL1, while MpRSL1 positively regulates MpFRH1 transcription, forming a negative feedback loop. MpRSL1 is a key positive regulator of rhizoid development in *M. polymorpha*. In the absence of this feedback loop (in *Mpfrh1* loss-of-function lines) the patterning of rhizoid initials, RIs, is disrupted, and the formation of RI clusters occurs more frequently, suggesting that the miRNA MpFRH1 restricts MpRSL1 expression to individual epidermal cells.

To determine where MpFRH1 - MpRSL1 interaction occurs, I investigated their promoter activity using promoter reporter fusions, their RNA accumulation pattern using *in situ* hybridization, as well as the MpFRH1 activity using a miRNA biosensor. I demonstrate that the *MpRSL1* promoter is active in epidermal cells around the apical notch where cell division and cell differentiation take place, and in RIs in the central regions of *M. polymorpha* gemmae. I detected MpFRH1 activity in the apical notch of young gemmae. These observations indicate that MpFRH1 restricts MpRSL1 expression in the vicinity of the apical notch where epidermal patterning occurs. This repression leads to the development of discrete rhizoid cells and suppresses the development of rhizoid clusters.

4.2 Introduction

Plant development depends on the generation of diverse cell types at a given position at a given time. The plant epidermis has been used as an excellent model system to study cell fate specification due to its planar structure, which is easy to access and to manipulate. The establishment and maintenance of [epidermal] patterning is one of the key questions for developmental biology. Considerable knowledge how patterning is controlled has been acquired by studying spatiotemporal regulation of gene interactions and cell behaviours. In animals, cell fates are determined by both positional information and cell lineage-dependent mechanism, whereas plant development is guided by position-based mechanisms and no evidence for lineage restriction has been reported to date (Rudel and Sommer, 2003; Pallakies and Simon, 2010; Costa, 2016; Yu et al., 2017). Position-based mechanisms rely on cell-cell communication to exchange positional information during development. Plant cells receive positional information from adjacent cells, which can also be transmitted to cells further away (e.g. short and long range transport of auxin, Robert and Friml, 2009). Cells typically receive signals from their neighbouring cells via receptors that are located in the plasma membrane. Receptor ligands that bind to the receptors to mediate signalling typically are hormones including small organic molecules, as well as larger peptides and small proteins (e.g. CLV3 – CLV1/2 interaction, Clark, 2001). Due to the existence of microscopic channels in the cell wall of connected cell, called plasmodesmata, plant cells can also exchange signals directly, including TFs or miRNAs (Chitwood and Timmermans, 2010; Vatén et al., 2011; Han et al., 2014). The versatile exchange of positional information among cells coordinates plant development.

An example of how positional cues control cell fate determination is the regulation of trichome development in the leaf epidermis of *A. thaliana*, which was already highlighted in chapter 3.5. In brief, a transcriptional complex consisting of the R2R3-Myb

TF GLABRA1 (GL1), the WD-40 scaffold protein TRANSPARENT TESTA GLABRA1 (TTG) and the bHLH TFs GLABRA3 (GL3) and ENHANCER OF GL3 (EGL3) activates the expression of the homeodomain TF GLABRA2 (GL2) which determines trichome cell fate initiation (Rerie et al., 1994). Consequently, this complex induces the transcription of redundantly acting inhibitors, e.g. CAPRICE (CPC), TRIPTYCHON (TRY), ENHANCER OF TRY (ETC1), ETC2, ETC3 and TRICHOME-LESS1 (TCL1). Once transcribed and translated, these inhibitor proteins move to the neighbouring cells via plasmodesmata, where they can counteract the activator complex by competing with GL1. TTG/GL3/EGL3 bound to a CPC/TRY protein suppresses the activation of GL2 transcription and hence trichome formation (Torii, 2012). Interestingly, while GL2 acts as an activator of specialised leaf epidermal cells, it is a suppressor of specialised root epidermal cells, e.g. root hair cells (Di Cristina et al., 1996; Masucci et al., 1996, reviewed by Kellogg, 2001; Schiefelbein, 2003; Pesch and Hülskamp, 2004; Ishida et al., 2008). The mobile characteristic of TRY (and CPC) during trichome formation (as well as root hair formation) represents only one example how cells communicate with each other by transmitting positional information to control cell differentiation.

In the liverwort *M. polymorpha* the epidermis harbours specialised structures such as unicellular rhizoid cells on their ventral surface, as well as multicellular air pore complexes, gemma cups and gemmae on their dorsal surface. While air pore complexes only develop once dorsoventrality is established in thalli developing from gemmae, rhizoid cells are already differentiated during gemma development as rhizoid initial cells (RIs) and can be identified on a mature gemma. Typically, RIs develop as isolated RIs or small groups of two or three cells, well spread across the epidermal surface. There is no evidence for a lineage dependent cell fate determination of rhizoid cells, and individual rhizoid cells are spaced from each other by epidermal cells (as I described in chapter 3). However, in

plants harbouring a loss-of-function mutation in the miRNA *MpFRH1* allele, the density and organisation of those RIs change significantly, causing the development of RI clusters (as I described in chapter 3). This indicates that *MpFRH1* could act as a miRNA propagating positional information among the epidermal cells to control rhizoid patterning. *MpFRH1* directly targets mRNA of *MpRSL1*, a bHLH TF that positively controls the development of rhizoid cells. RIs of *MpRSL1*^{GOF} gemmae grow also in clusters, like those in *Mpfrh1*^{LOF} gemmae, which indicates that the interaction between *MpFRH1* and *MpRSL1* governs RI patterning during gemma development (chapter 2).

Since the promoter of *MpFRH1* is predominantly active in RIs and the function of the mature miRNA is presumably the suppression of *MpRSL1* mRNA accumulation in non-RI cells (chapter 2), I hypothesise that *MpFRH1* acts as a mobile miRNA that radiates from RIs into non-RIs to restrict the expression of *MpRSL1* to individual epidermal cells and hence their differentiation into rhizoid cells. Alternatively, the miRNA *MpFRH1* might act cell autonomously within growing rhizoid cells to control levels of *MpRSL1* mRNA and prevent *MpRSL1* movement into adjacent epidermal cells.

To understand how *MpFRH1*-*MpRSL1* interaction controls rhizoid patterning, I investigated the spatial expression pattern of both, *MpRSL1* and *MpFRH1*. First, I tested where the promoter of *MpRSL1* and *MpFRH1* is active, followed by investigation of the spatial activity of *MpFRH1* and ultimately the transcript accumulation of *MpRSL1* mRNA. This would allow me to see where *MpRSL1* transcription takes place, where *MpFRH1* is active and eventually where *MpRSL1* mRNA accumulation occurs. Determining where *MpFRH1* is active will help me to address the hypothesis if *MpFRH1* acts non-cell autonomously in epidermal cells next to RIs.

4.3 Results

4.3.1 The *MpRSL1* promoter is active in the region including and around the apical notch and in RIs in the central region of gemmae

To test where *proMpRSL1* is active and *MpRSL1* transcription occurs, I generated a *MpRSL1* promoter reporter line. I amplified the genomic region 5kb upstream of the *MpRSL1* start codon and fused this fragment in front of a fluorescent reporter gene, a triple YFP protein harbouring a nuclear localisation sequence. I transformed the vector harbouring this construct and a hygromycin resistance gene into *M. polymorpha* spores and selected for hygromycin resistant and YFP expressing transformants. I analysed the expression pattern of the *MpRSL1* promoter in 0-day-old gemmae. In Figure 4.1, the promoter activity of *MpRSL1* in three independent lines is displayed. Individual cells of gemmae were characterised using the autofluorescence of the chlorophyll (Fig 4.1, panel 1), allowing the identification of RIs and non-RIs. The YFP signal represents the promoter activity of *MpRSL1* (Figure 4.1, panel 2). Comparison of the YFP signal and the auto fluorescing signal (Figure 4.1, panel 3) allows the localisation of the *MpRSL* promoter activity within the epidermis of 0-day-old gemmae.

proMpRSL1:3xNLS-YFP#1.1 and *proMpRSL1:3xNLS-YFP#2.1* show a very similar expression pattern, with high promoter activity in the apical notches (Figure 4.1, D + H), where cell division and cell differentiation take place. Towards the centre of the gemmae, the promoter activity decreases, except in RIs, where the YFP signal is stronger than in surrounding epidermal cells. *proMpRSL1:3xNLS-YFP#1.17* (Figure 4.1, I-L) shows weaker promoter activity in all cells than *proMpRSL1:3xNLS-YFP#1.1* and *proMpRSL1:3xNLS-YFP#2.1*. High promoter activity was observed in a few cells in the apical notch, while weak promoter activity was observed in RIs and individual epidermal cells. Even though

the promoter activity was lower in *proMpRSL1:3xNLS-YFP#1.17* than in *proMpRSL1:3xNLS-YFP#1.1* and *proMpRSL1:3xNLS-YFP#2.1*, the same expression pattern was observed, with higher activity around the apical notch which becomes more specified towards the centre of the gemmae. Based on the promoter expression in those three independent lines, I conclude that *proMpRSL1* is preferentially active in cells surrounding the apical notch, where cell division and cell differentiation take place, as well as in individual epidermal cells, in particular RIs, in the centre of the gemmae, where rhizoid differentiation is established. This suggest that *MpRSL1* is ubiquitously transcribed in undifferentiated tissue (around the meristems) and during rhizoid cell differentiation *MpRSL1* transcription becomes more restricted to RIs only.

While the promoter activity of *proMpRSL1* was detected around the apical notch as well as in individual RIs, Honkanen et al. (2018) showed that the promoter activity of *MpFRH1* is particularly high in RIs as well as in mucilage papillae cells. I confirmed this finding by imaging *proFRH1:3xNLS-YFP#19* (Fig 4.1, M-P; provided by Suvi Honkanen), where YFP signal was high in RIs and mucilage papillae cells. This activity profile suggests that *MpFRH1* miRNA is only transcribed in RIs that already established its cell fate as well as in mucilage papillae cells surrounding the apical cell.

The promoter activity of *MpRSL1* and *MpFRH1* suggests that *MpRSL1* is transcribed from early development (in the region of the meristem) through to late development in differentiating cells. By contrast, *MpFRH1* transcription is restricted to already differentiated, specialised epidermal cells, e.g. RIs and mucilage papillae cells. This suggest that *MpRSL1* is active pre-cell fate differentiation, acting upstream of *MpFRH1*. Only later, during cell differentiation, both *MpRSL1* and *MpFRH1* are transcribed in the same specialised epidermal cells.

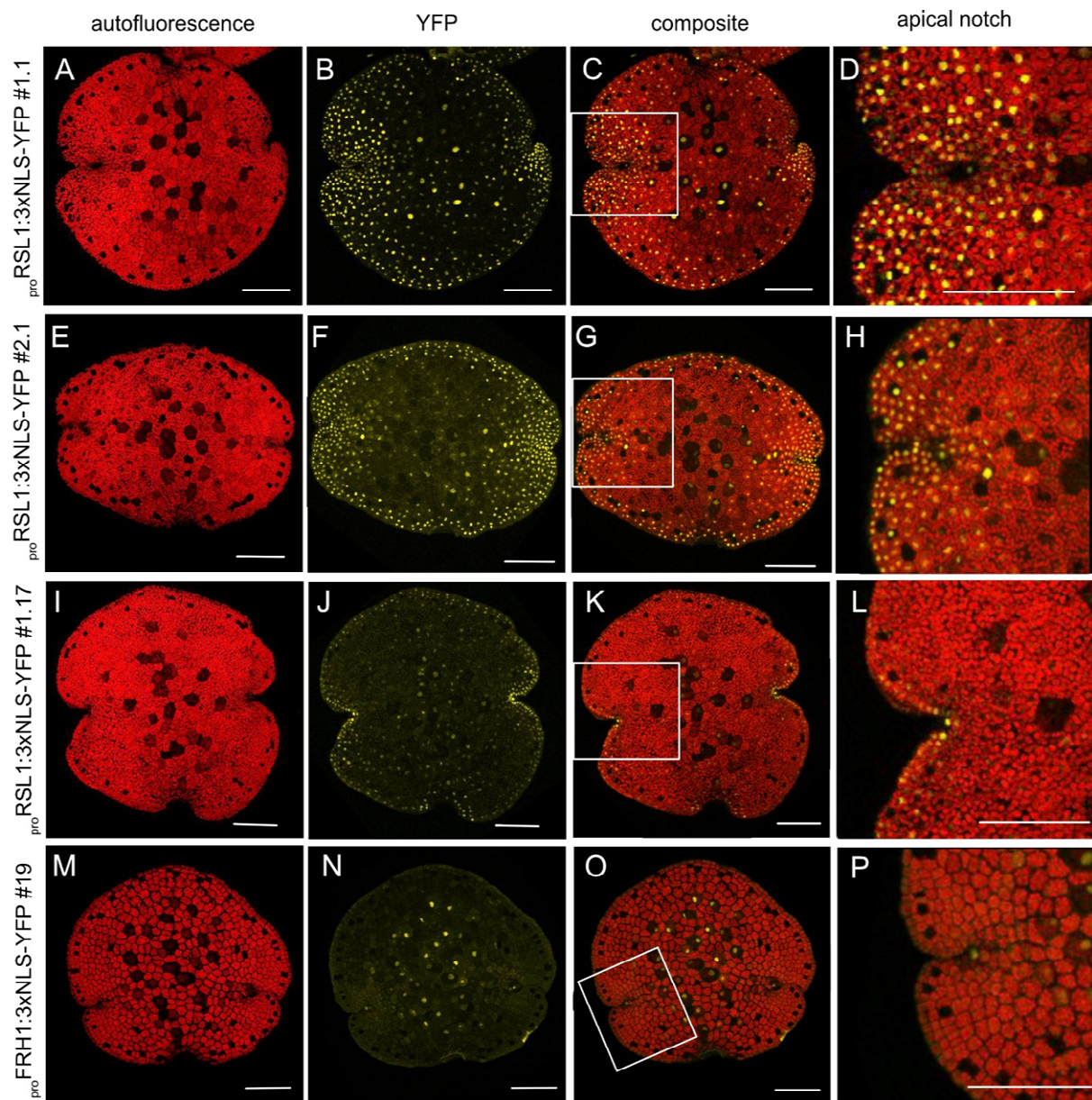


Figure 4.1 *proMpRSL1* and *proMpFRH1* activity in 0-day-old gemmae

Confocal images of 0-day-old gemmae transformed with *proMpRSL1:3xNLS-YFP* in three independent lines (#1.1, A-D; #2.1, E-H; #1.17, I-L) and *proMpFRH1:3xNLS-YFP* (#19, M-P). First column autofluorescence of chlorophyll, second column YFP fluorescence, third and fourth column overlay of both signals. Forth column shows a close-up of the apical notch highlighted in C, G, K and O (white square). Scale bar = 100 μ m

4.3.2 An MpFRH1 sensitive reporter gene can be used to visualize MpFRH1 activity

The promoter of *MpFRH1* is predominantly active in RIs and mucilage papillae cells around the meristem, indicating that *MpFRH1* is transcribed in those specialised epidermal cells. *MpRSL1* mRNA is a direct target of MpFRH1 and the promoter of *MpRSL1* is broadly active around the apical notch and more restricted to RIs towards the centre of a gemmae. Since the *MpFRH1* promoter is active in RIs and mucilage papillae cells and *MpRSL1*^{GOF} and *Mpfrh1*^{LOF} develop clusters of RIs and mucilage papillae cells (only rhizoid clusters shown for *MpRSL1*^{GOF}), I propose that MpFRH1 may act non-cell autonomously, I suggest that it moves from those specialised epidermal cells outwards to repress *MpRSL1* accumulation in neighbouring cells, and hence further specialisation of those epidermal cells (into rhizoid or mucilage papillae cells).

To test if indeed MpFRH1-directed cleavage of *MpRSL1* mRNA occurs in cells that in which MpFRH1 is not expressed, I generated an MpFRH1 sensitive biosensor. This biosensor expresses a fluorescent reporter gene (YFP) under the ubiquitously active *MpEF1α* promoter. This reporter gene is fused to the MpFRH1 target site sequence (Figure 4.2A). In this way the expression of the reporter gene depends on the activity of miRNA MpFRH1. In cells where no MpFRH1 activity occurs, YFP signal will be expressed due to the ubiquitous activity of *proMpEF1α*. In cells where MpFRH1 is active, *YFP* mRNA is targeted by MpFRH1 and YFP expression will be reduced. To test for *proMpEF1α* activity, I generated a control construct with the *proMpEF1α* driving YFP without the MpFRH1 target site (Figure 4.2 F). In cells harbouring this construct, YFP signal should be detectable in all cells, independent of MpFRH1 activity. By comparing the expression of the reporter gene with and without MpFRH1 target site, I can infer where MpFRH1 directed post transcriptional silencing occurred. I hypothesise that MpFRH1 is a non-cell autonomous

miRNA, causing post transcriptional silencing in cells neighbouring developing RIs or mucilage papillae cells. If this hypothesis is correct, I expect to observe (i) a lower expression of the reporter genes in epidermal cells surrounding RIs or MPCs compared to epidermal cells without neighbouring RIs or MPCs or (ii) a lower level of activity in cells in which pattern formation is taking place and MpFRH1 is repressing MpRSL1. Expression of the resistant reporter gene should not be affected in epidermal cells with or without neighbouring RIs/MPCs. The analyses of the generated biosensors allowed me to test these hypotheses.

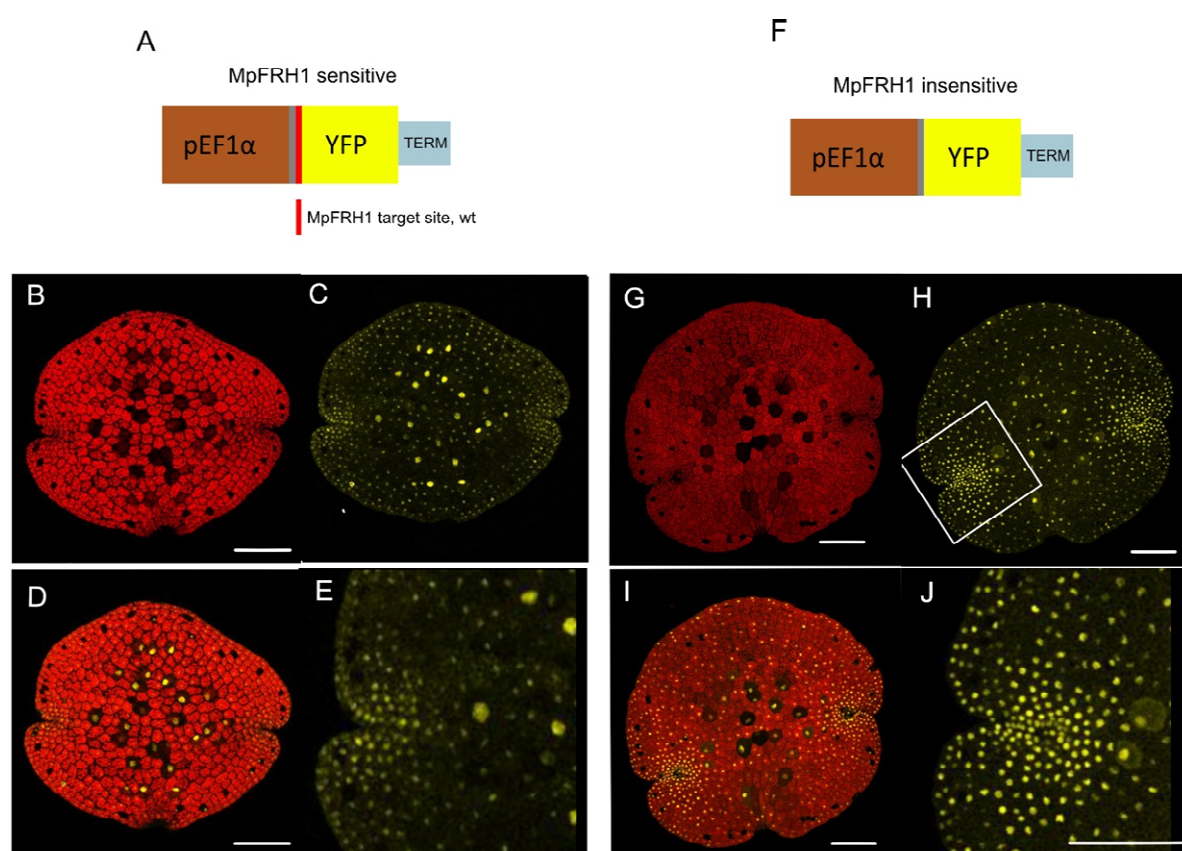


Figure 4. 2 Construct and expression pattern of a MpFRH1 biosensor in 0-day-old gemmae

A) Design of MpFRH1 sensitive biosensor with *proMpEF1α* expressing a *NLS-YFP* reporter gene, harbouring the 21 bp long MpFRH1 target site and a terminator sequence

B-E) Expression pattern of the MpFRH1 sensitive biosensor in 0-day-old gemmae showing autofluorescence of chlorophyll (B), YFP signal (C) and both signals together (D). E) shows the YFP signal within the apical notch highlighted in C (white square)

F) Design of MpFRH1 resistant biosensor with *proMpEF1α* expressing a *NLS-YFP* reporter gene without the 21 bp long MpFRH1 target site, but a terminator sequence

F-J) Expression pattern of the MpFRH1 resistant biosensor in 0-day-old gemmae showing autofluorescence of chlorophyll (G), YFP signal (H) and both signals together (I).

J) shows the YFP signal within the apical notch highlighted in H (white square)

Scale bar: 100µm, grey box : NLS

4.3.3 MpFRH1 supresses the expression of its target gene in cells around the meristem while no MpFRH1 activity was detected in epidermal cells surrounding differentiated RIs

To visualise MpFRH1 activity I transformed an MpFRH1 sensitive and an MpFRH1 resistant reporter gene into wildtype spores. Positive transformants were identified based on their resistance to hygromycin. YFP expression in 0-day-old gemmae was compared to each other to localise MpFRH1 activity. YFP expression in MpFRH1 resistant lines appears to be abundant among the entire gemmae with high activity around the apical notches (Figure 4.2 F, G, and H). In MpFRH1 sensitive lines, the YFP signal appears to be high in RIs and reduced in epidermal cells and apical notches (Figure 4.2 B, C, and D). This indicates that MpFRH1 is causing post transcriptional silencing of its target gene around the apical notch and in epidermal cells surrounding RIs.

To prove that there is a reduction of YFP signal around the apical notch and/or epidermal cells, I quantified the relative YFP signal intensity in cells around the meristem in epidermal cells surrounding 1, 2 or 3 RIs and in RIs itself in MpFRH1 sensitive and MpFRH1 resistant lines (three independent lines each).

MpFRH1 reduced YFP signal around the meristem

First, I quantified the maximum pixel value of the image. For further quantification I selected images that do not exceed a maximum pixel value of 255 (maximal pixel value indicates overexposure) or if only individual pixel were saturated. The maximal pixel value was used to quantify the relative pixel intensity of YFP signal of interest.

I selected 25 to 30 nuclei surrounding the apical notch and measured their brightest YFP signal using the multi point tool in ImageJ (see Figure 4.3 B). I then calculated the average YFP signal of those cells in at least 5 biological replicates (except for line 1.9 of

the MpFRH1 sensitive line, where only three replicates were quantified). The relative YFP signal in MpFRH1 sensitive lines was on average 0.33 (SD = 0.03), while the relative YFP signal in MpFRH1 resistant lines was up on average 0.49 (SD = 0.09) (Figure 4.3A). The relative reduction of YFP signal around the apical notch in MpFRH1 sensitive lines indicates that the miRNA MpFRH1 is mediating post transcriptional silencing of its target gene, *MpRSL1*, within the apical notch.

This finding is supported by measuring the relative YFP intensity in a manually set area of approximately 3x3 cells next to the predicted apical cell (Figure 4.3 D). I quantified the maximal YFP signal intensity within that area in MpFRH1 sensitive and MpFRH1 resistant lines. The maximal intensity in MpFRH1 sensitive lines is on average 0.56 (SD = 0.04) and in MpFRH1 resistant lines the YFP intensity is on average 0.71 (SD = 0.16) (Figure 4.3 C). The reduction of the maximal YFP signal in MpFRH1 sensitive lines around the apical notch is consistent with the previously shown reduction of the average YFP signal in cells around the meristem, which suggests that MpFRH1 is active within the apical notch of young gemmae.

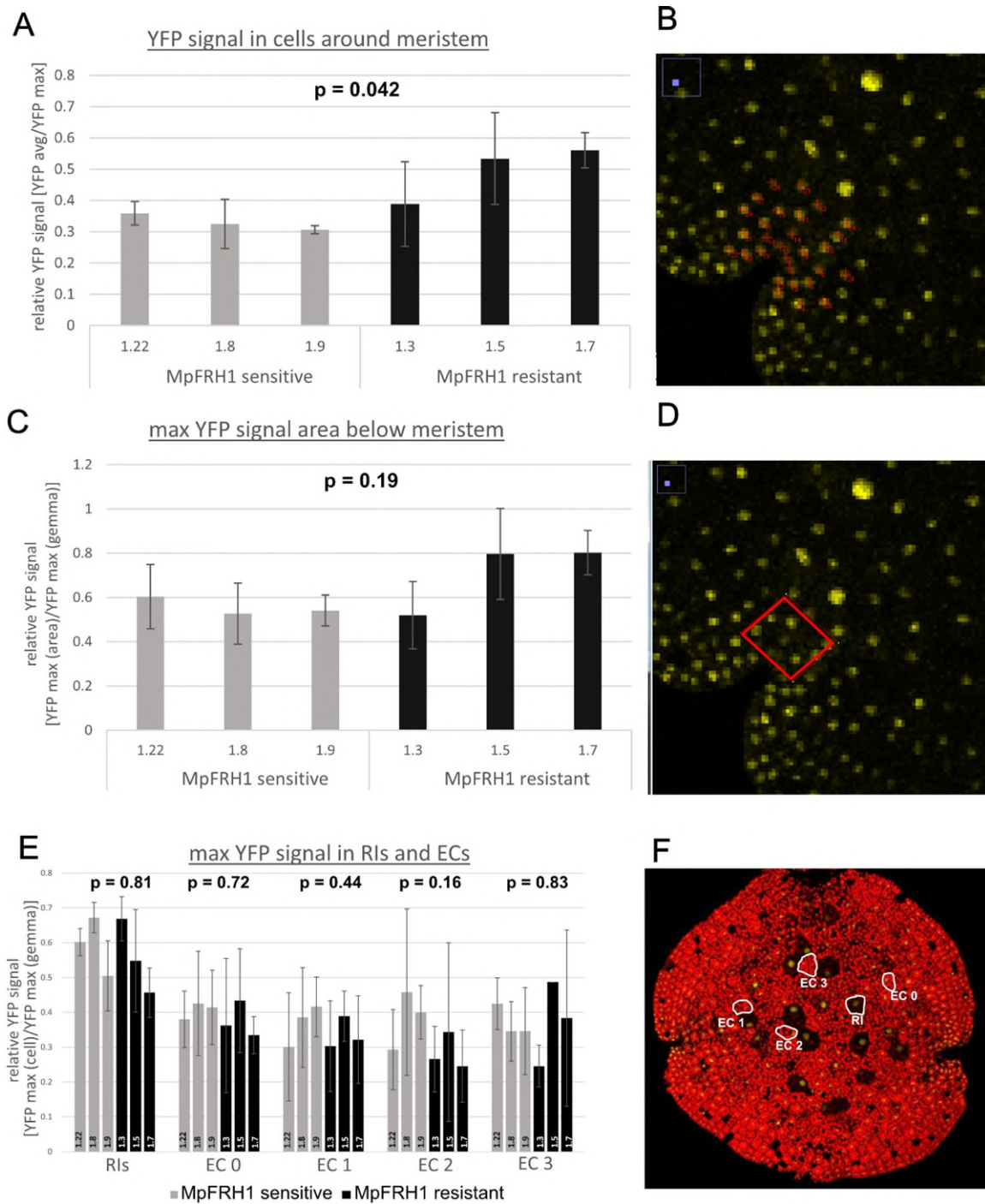


Figure 4. 3 YFP signal intensity in 0-day-old gemmae of MpFRH1 sensitive and MpFRH1 resistant lines

A) Relative YFP signal in 25-30 measured cell nuclei around the apical notch (as shown in B), in three MpFRH1 sensitive and three MpFRH1 resistant lines. Statistical significance between the three MpFRH1 sensitive and the three MpFRH1 resistant lines based on student t-test, $p=0,042$.

C) Relative YFP signal in a 3x3 cell grid next to the apical notch towards the gemma centre (as shown in D) in three MpFRH1 sensitive and three MpFRH1 resistant lines. Statistical significance between the three MpFRH1 sensitive and the three MpFRH1 resistant lines based on student t-test, $p=0,19$ (not significant).

E) maximal YFP signal in RIs and epidermal cells (ECs, with 0, 1, 2 or 3 neighbouring RIs (EC 0, EC 1, EC 2, EC 3, respectively – as demonstrated in F) in three MpFRH1 sensitive and three MpFRH1 resistant lines.

Statistical significance between MpFRH1 sensitive and MpFRH1 resistant lines based on student t-test for RIs, EC 0, EC 1, EC 2 and EC 3 given above bars ($p>0.05$, not significant)

MpFRH1 resistant lines in grey (n [gemmae]): #1.22(n=9), #1.8 (n=5), #1.9 (n=3),

MpFRH1 resistant lines in black (n [gemmae]) :#1.3 (n=9), #1.5 (n=5), #1.7 (n=6)

A different biosensor supports the hypothesis that MpFRH1 acts in apical notches

The observation of a reduced fluorescent signal around the apical notch made in gemmae harbouring the MpFRH1 miRNA biosensor (Figure 4.2 and Figure 4.3) is consistent with findings observed in gemmae harbouring similar biosensor constructs. I generated additional variants of biosensors by using different promoters and different reporter genes. One alternative biosensor construct is composed of the ubiquitously active *proOsACTIN* which is driving the expression of three MpFRH1 sensitive CYAN FLUORESCENT PROTEINS (CFPs) and a second *proOsACTIN* driving the expression of three MpFRH1 resistant YFPs (Figure 4.4 A[I], B-F). Therefore, the YFP signal is ubiquitously expressed (representing *proOsACTIN* activity) while cells that lack the CFP signal are cells in which MpFRH1 is active. In 0-day-old gemmae the CFP signal is highly reduced in the apical notches while YFP is present (Figure 4.4 B-G). Since the CFP signal is reduced while the YFP signal is present it indicates that MpFRH1 causes the reduction of CFP expression around the apical notch (Figure 4.2). At the centre of the gemma the YFP expression is much weaker relative to their apical notches while the CFP signal is detectable. The strong CFP expression in RIs and non-RIs among in the central region suggests that MpFRH1 does act in the central region where cells are already differentiated, which is consistent with observations in the above described biosensor. However, since co-localisation of YFP and CFP is not possible (Figure 4.4 B-G) and CFP and YFP show a mutually exclusive expression pattern, it is possible that co-expression of two reporter genes under the same promoter causes mutual silencing. This would imply that only one of these promoters (*proOsACTIN*) is active.

The hypothesis that the promoter activity can be silenced when the same promoter has been used twice within a transgene is reinforced due to findings of the expression pattern of another biosensor. The third variant of a biosensor I generated, harbours two *MpEF1 α* promoters. One *proMpEF1 α* was used to express a single MpFRH1 sensitive YFP and a second *proMpEF1 α* to express a single MpFRH1 resistant CFP (Figure 4.4 A [II], H-M). Here only YFP signal and no CFP expression was detected, indicating that the second *MpEF1 α* promoter is silenced and only one promoter is active. No conclusions can be made about MpFRH1 activity using this biosensor.

Based on findings that the promoter of MpFRH1 is active in mucilage papillae cells (section 4.3.1) and that MpFRH1 reduces the expression of its target gene within the apical notches (section 4.3.2), I hypothesis that MpFRH1 is generated in mucilage papillae cells and causes post transcriptional silencing of its target gene, *MpRSL1*, within the apical notch. If MpFRH1 acts cell autonomous or non-cell autonomous cannot be answered with that analysis. However, since I quantified the relative YFP signal of up to 30 cells within the apical notch, I presume the reduction of YFP is caused by a rather broad activity of MpFRH1 and not only due to the reduction within the mucilage papillae cells, which indicates that MpFRH1 acts non-cell autonomous in the apical notch of young gemmae.

A

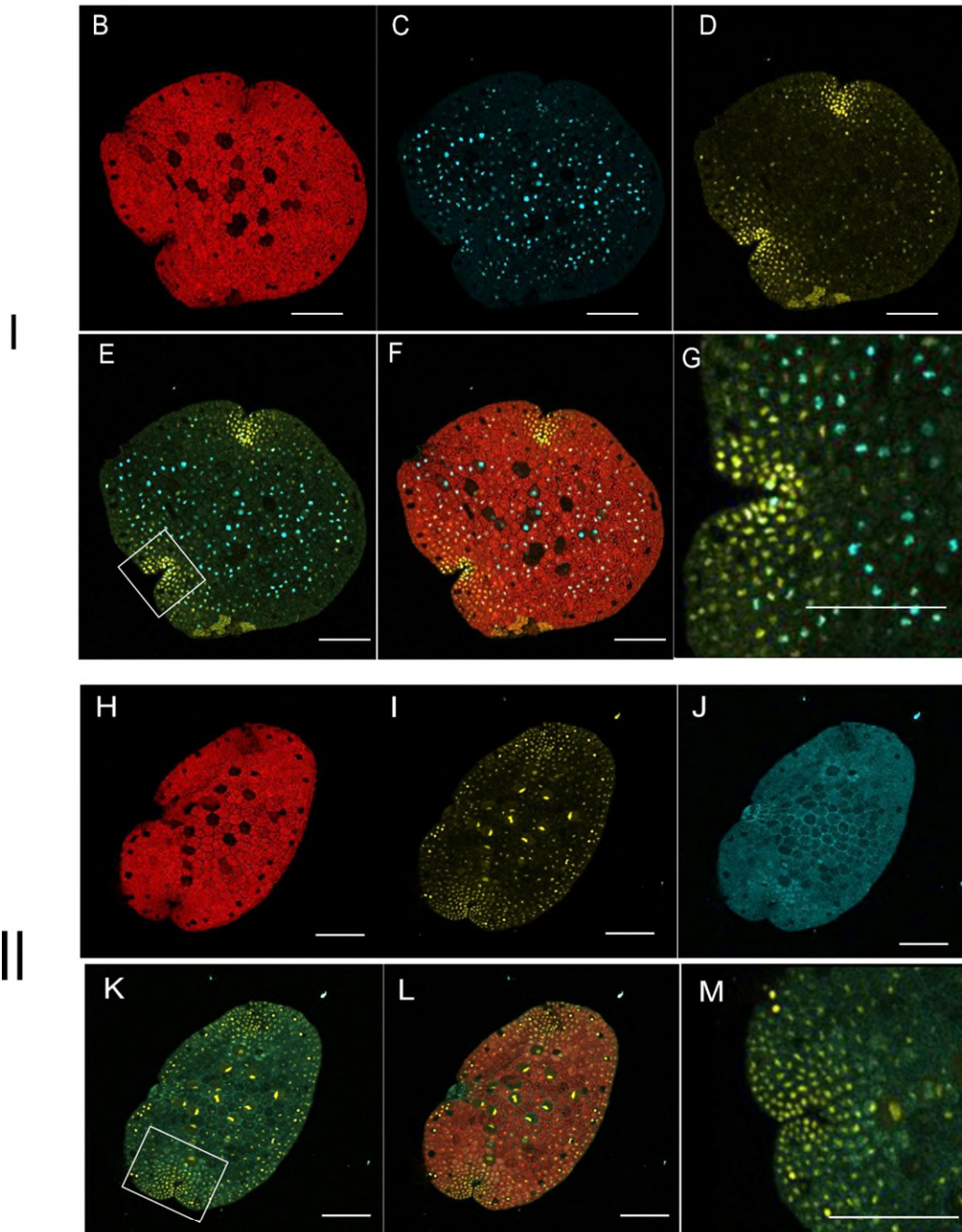
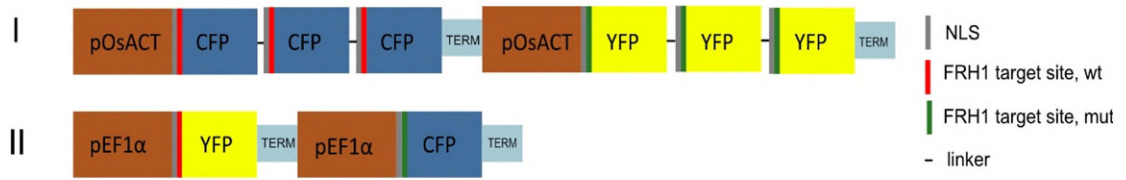


Figure 4. 4 Constructs and expression pattern of two further MpFRH1 activity biosensors in 0-day-old gemmae

A) I: Design of MpFRH1 activity biosensor with *proOsACTIN* expressing three *NLS-CFP* reporter genes harbouring the 21 bp long MpFRH1 target site and a terminator sequence followed by a second *proOsACTIN* expressing three *NLS-YFP* reporter genes without the 21 bp long MpFRH1 target site and a terminator sequence. II: design of MpFRH1 activity biosensor with *proMpEF1α* expressing a *NLS-YFP* reporter gene harbouring the 21 bp long MpFRH1 target site and a terminator sequence followed by a second *proMpEF1α* expressing a *NLS-CFP* reporter genes with a 21 bp long sequence that is resistant to MpFRH1 activity and a terminator sequence.

B-G) Expression pattern of the MpFRH1 sensitive biosensor in 0-day-old gemmae showing autofluorescence of chlorophyll (B), CFP signal (C), YFP signal (D), CFP and YFP signal together (E) and all three signals together (F). G shows the apical notch highlighted in E

H-M) Expression pattern of the MpFRH1 sensitive biosensor in 0-day-old gemmae showing autofluorescence of chlorophyll (G), YFP signal (H), CFP signal (I), CFP and YFP signal together (J) and all three signals together (K). M shows the apical notch highlighted in K

Scale bar: 100µm, grey box : NLS

MpFRH1 is not active in mature RIs or in epidermal cells surrounding RIs

The promoter of *MpFRH1* is active in RIs ($_{pro}MpFRH1:3xNLS-YFP$ #19 as shown in Figure 4.1 M-P), suggesting that *MpFRH1* is transcribed in those cells and either cause post transcriptional silencing of its target *MpRSL1* in those cells, or, if it acts non-cell autonomously, in epidermal cells surrounding RIs. To test if *MpFRH1* causes the suppression of its target gene in RIs or in epidermal cells surrounding RIs, I quantified the relative YFP signal intensity in RIs and in epidermal cells with 0,1,2 or 3 neighbouring RIs in three *MpFRH1* sensitive and *MpFRH1* resistant lines (Figure 4.3, F). If *MpFRH1* acts cell autonomously, the relative YFP signal in RIs of *MpFRH1* sensitive lines would be reduced compared to *MpFRH1* resistant lines. If *MpFRH1* acts non-cell autonomous and moves from RIs outwards into neighbouring epidermal cells to cause post transcriptional silencing in those cells, the YFP signal in epidermal cells of *MpFRH1* sensitive lines would decrease the more RIs are connected to the respective epidermal cell.

The relative YFP signal in RI of *MpFRH1* sensitive lines was on average 0.59 (SD=0.07, n=3) while in *MpFRH1* resistant lines it was 0.56 (SD=0.11, n=3) (Figure 4.3E). Hence there was no difference in YFP signal which indicates that no *MpFRH1* activity was detected in RIs. Also, in epidermal cells the YFP signal did not differ between *MpFRH1* sensitive and *MpFRH1* resistant lines. In epidermal cells without neighbouring RIs, the relative YFP signal was 0.41 (SD=0.04, n=3) and 0.38 (SD=0.05, n=3) in *MpFRH1* sensitive and *MpFRH1* resistant lines, respectively. With increasing number of neighbouring RIs the relative YFP signal did not significantly decrease, neither in *MpFRH1* sensitive nor in *MpFRH1* resistant lines. In *MpFRH1* sensitive lines the YFP signal was on average 0.37 (SD=0.05), 0.38 (SD=0.08) and 0.37 (SD=0.05) for 1, 2 and 3 neighbouring RIs, respectively. For *MpFRH1* resistant lines the YFP signal was on average 0.34 (SD=0.05), 0.28 (SD=0.05) and 0.37 (SD=0.12) for 1,2 and 3 neighbouring RIs. Since the

relative YFP signal did not decrease in epidermal cells with increasing number of neighbouring RIs (Figure 4.3.E), it suggests that MpFRH1 is not active in either RIs or in epidermal cells next to differentiated RIs.

Taken together those results indicate that MpFRH1 causes post transcriptional silencing of its target gene within the apical notch and not in epidermal cells in the centre of young gemmae. It is possible that the YFP signal detected in mature RIs of *proMpFRH1* reporter lines are remains of the YFP protein from young developing RIs, and therefore do not represent active transcription.

4.3.4 Establishing *in situ* hybridization (ISH) of young gemmae to localise MpRSL1 mRNA

The promoter of MpRSL1 is dynamically active, with high activity around the apical notch, RIs and marginal epidermal cells and low activity in non-specialised epidermal cells, indicating the spatial accumulation of MpRSL1 transcripts. However, post-transcriptional gene silencing mechanisms, in particular MpFRH1 directed degradation of MpRSL1 mRNA, are likely to alter the spatial distribution of MpRSL1 mRNA. The results of the biosensor indicate that MpFRH1 is acting in the apical notch to cause post transcriptional gene silencing of its target MpRSL1 there. If MpFRH1 indeed causes degradation of MpRSL1 mRNA within the apical notch, I expect MpRSL1 mRNA to be less abundant in the apical notch of wildtype gemmae and more abundant in apical notches of *Mpfrh1^{LOF}*.

To visualise MpRSL1 mRNA accumulation, I performed whole mount ISH on young gemmae as well as ISH on sections of young gemmae. This included the identification of a suitable positive control, a gene that is transcribed ubiquitously in young gemmae. I tested *INCOMPLETE ROOT ELONGATION* (MpIRE), a protein kinase, which promoter is active in all epidermal cells in young gemmae (Honkanen et al., 2018). As a

second positive control I used *GLABRA3* (MpGL3), a bHLH domain protein, which was successfully used in the Dolan lab during whole mount ISH of 2-week-old thalli (Catarino, 2017). Differences of the MpRSL1 promoter activity profile and MpRSL1 mRNA accumulation, will indicate where post transcriptional silencing mechanisms operate and where MpFRH1 is likely to be active. Ultimately, comparing MpRSL1 mRNA accumulation in wildtype with Mpfrh1^{LOF} gemmae will reveal the localisation of MpFRH1 activity.

While whole mount ISH did not yield conclusive accumulation pattern between antisense (illustrating mRNA accumulation of MpRSL1, MpIRE, MpGL3) and sense (negative control) probes, the best results were observed using ISH on sections, which are discussed in the following paragraph.

ISH on sections of 6-hours old gemmae testing MpIRE, MpGL3 and MpRSL1 mRNA accumulation

To visualise the localization of MpRSL1 mRNA in young gemmae, 6 hours grown gemmae were fixed in 4%PFA, wax embedded and sectioned. ISH was performed on 8µm thick sections using 100-250 bp long MpRSL1 DIG-labelled probes as well as MpIRE and MpGL3 probes as positive controls at same conditions.

In gemmae treated with DIG-labelled RNA antisense probes of MpIRE and MpGL3 a positive signal (in the form of a purple/blue colour development) can be observed in individual epidermal cells, potentially comprising RIs (Figure 4.5 A,B,E,F). Generally no colour development is observed in gemmae treated with DIG labelled RNA sense probes (Figure 4.5 C,D,G,H). Occasionally individual epidermal cells of MpIRE sense treated sections show similar staining pattern to the antisense treated samples, but this staining is usually weaker (Figure 4.5 D). This indicates that MpIRE and MpGL3 mRNA accumulates predominantly in those RIs. A restricted accumulation of MpIRE and MpGL3 mRNA does

not conform with the expected accumulation pattern, since the promoter of *MpIRE* and *MpGL3* is uniformly active in all epidermal cells (Honkanen et al., 2018, Catarino, 2017), hence mRNA accumulation is expected to be rather ubiquitous. There are several possibilities which may account for the discrepancy between promoter activity and detected mRNA accumulation of *MpIRE* and *MpGL3*. It is possible that emerging rhizoid cells have different properties compared to non-specialised epidermal cells, e.g. endogenous phosphatase activity or histological properties that cause a stronger colour development or a more efficient probe penetration into those cell types. Or indeed, mRNA of *MpIRE* and *MpGL3* particularly accumulates in RIs and post transcriptional modifications restricts their accumulation to this cell type.

A similar pattern can be observed for *MpRSL1*, where a rather faint signal can be detected in individual RIs of gemmae treated with DIG labelled *MpRSL1* antisense RNA (Figure 4.5 I, J). This observation indicates that *MpRSL1* mRNA accumulates predominantly in RIs. However, due to a weak signal intensity, it suggests that only low transcript accumulation occurs. This observation is not very well supported since gemmae treated with *MpRSL1* sense probe show some pigmentations in individual cells as well, which is not clearly distinguishable from the staining observed in *MpRSL1* antisense probes (Figure 4.5 K, L). There are no indications for *MpRSL1* mRNA accumulating in the apical notch. No colour development can be observed at the margin of the generated sections. This can either be due to technical issues, that sections do not show the apical notch or due to biological reasons, that indeed *MpRSL1* mRNA does not accumulate in apical notches.

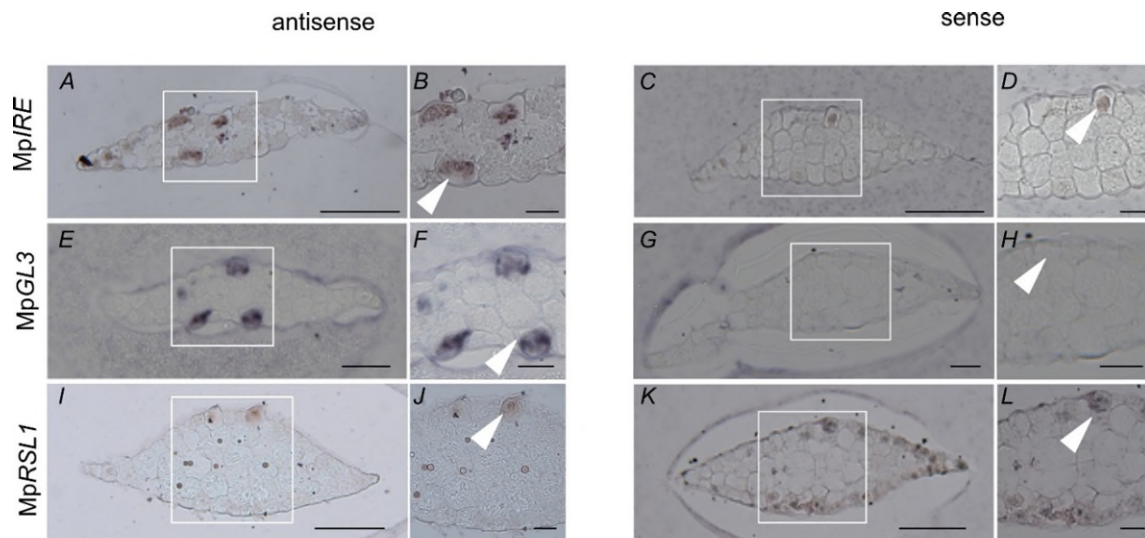


Figure 4. 5 ISH on sections of 6-hours-old gemmae for *MpIRE*, *MpGL3*, *MpRSL1*
 Colour development after ISH of gemmae treated with antisense and sense probes for *MpIRE* (A-D), *MpGL3* (E-H) and *MpRSL1* (I-L). Scale bar = 100 μ m in A, C, E, G, I, K and 25 μ m in B, D, F, H, J, L. Arrowheads highlight cells, presumably RIs, with stronger staining.

Overall the results from carrying out ISH on sections are not conclusive and need to be treated with caution. There is a general trend of RIs to be stained, independent of the probes used, indicating that indeed those cells are rich in transcripts or that those cell types are prone to be stained, due to histological properties allowing probes to enter those cells to a much higher extent than other epidermal cells. Establishing a positive control, which shows staining of other cell types than RIs, would be essential to convincingly state that *MpRSL1* mRNA accumulates predominantly in RIs. A possibility is the generation of YFP overexpressing lines, and designing probes for YFP mRNA, which is likely to be resistant to post transcriptional silencing mechanisms. The presence of YFP proteins in non-rhizoid cells can be confirmed using fluorescence microscopy.

I also tested ISH on whole mount gemmae to localize mRNA accumulation of *MpRSL1*, *MpIRE* and *MpGL3* among the entire epidermal surface of young gemmae. Staining of antisense and sense treated samples were not distinguishable and hence no conclusive accumulation pattern could be extracted from those experiments. In the following paragraphs I summarize the results of three independent whole mount ISH.

Whole mount ISH of 0-day-old gemmae (WH-ISH I)

The first attempt to visualise *MpRSL1* mRNA accumulation using ISH was performed on gemmae that were grown for 4 hours. DIG-labelled probes of *MpIRE* and *MpRSL1* were hydrolysed to fragments of 250-550 bp length. I used approximately 100ng of sense and antisense probe when hybridising probes to mRNA. Gemmae treated with sense and antisense probe of *MpIRE* did not show any signal and no colour development was visible (Figure 4.6B-C). No distinct staining was observed, which could be due to technical issues, e.g. probe permeability, fixation of the gemmae, degradation of the probes or due to biological reasons, e.g. *MpIRE* mRNA does not accumulate in 4hour old gemmae, which is however contradictory to its transcriptional activity (Figure 4.3 B,C).

MpRSL1 mRNA accumulation was observed in individual epidermal cells in the centre of the gemmae as well as in individual cells at the margin of a gemmae. Those individual cells are potentially RIs, due to their size and position in the central region of the gemmae, and oil bodies, due to their smaller size at the margin of the gemmae with a regular spacing known from oil bodies (Figure 4.3 H). In most epidermal cells, no accumulation was observed. In gemmae that were treated with the sense probe, individual central and marginal epidermal cells showed colour development, similar to cells of gemmae treated with the antisense probe (Figure 4.3 I). Due to the similar staining profile

in both the sense and antisense treatments, it is not possible to draw conclusions about the *MpRSL1* mRNA accumulation pattern.

Since gemmae are a vegetative propagule with arrested growth until activation (usually once they are in contact with a substrate), low transcriptional activity is expected in gemmae until the point of activation (Eklund et al., 2015). For the next attempt to visualise *MpRSL1* mRNA, I decided to let the gemmae grow for 24 hours to ensure that activation had started before processing as well as increasing the quantity of applied probes to the maximal recommended volume of 14 µl, irrespectively of their concentration. Hence, the sense probe corresponded to approximately 750 ng and antisense probe to approximately 150 ng.

Whole mount ISH of 1-day-old gemmae using MpIRE and MpRSL1 (WM-ISH II)

The second attempt was performed using gemmae that were grown for 24 hours in liquid M51C medium. I increased the amount of probe that was added to the hybridization solution to the maximum that was recommended based on Althoff et al. (2014), namely 14 µl, irrespectively of its concentration based on the dot blot analysis (e.g. 150 ng of antisense and 750 ng of sense probe). Again, almost no staining was observed for gemmae incubated with *MpIRE* antisense probe, while colour development around the apical notch appeared in *MpIRE* sense probes. Similarly, *MpRSL1* antisense samples showed very weak colour development in RIs and around apical notches while the signal in *MpRSL1* sense probes is already detectable around the apical notches and in RIs. The early occurring strong staining in gemmae treated with the sense probe might be due to its higher concentration used, e.g. 750 ng, compared to antisense probes, e.g. 150 ng.

These results do not allow us to draw conclusions about the mRNA accumulation of *MpRSL1* or *MpIRE*, either due to technical issues or biological issues of *MpRSL1* and *MpIRE1* mRNA not being accumulated at a detectable level in 1-day-old gemmae.

Whole mount ISH of 1-day-old gemmae using MpGL3 and MpRSL1 (WM-ISH III)

The first two attempts of whole mount ISH were performed on young gemmae using *MpIRE* as a positive control and *MpRSL1* as gene of interest. No accumulation of *MpIRE* was detected, indicating that either *MpIRE* mRNA does not accumulate in very young gemmae, or that due to technical issues the accumulation of mRNA cannot be visualised under the used conditions. Due to similarities between gemmae treated with *MpRSL1* sense and *MpRSL1* antisense probes, no conclusive accumulation pattern can be deduced for *MpRSL1* mRNA.

Due to the lack of a clear signal in cells of very young gemmae treated with *MpIRE* antisense probe, a suitable positive control demonstrating that transcript accumulation can be visualised under the applied conditions is still missing. Therefore I decided to look for an alternative gene with known transcriptional activity during gemmaling development. *MpGL3* is a bHLH domain protein that was successfully used for ISH assays in 2-week-old thalli in *M. polymorpha* (Catarino, 2017). In the work described by Catarino (2017), the accumulation of *MpGL3* mRNA occurred on the dorsal side of the thallus and is absent at the ventral side. Catarino also reports that the promoter of *MpGL3* is ubiquitously active in 0-day-old gemmae, suggesting accumulation of *MpGL3* mRNA in all epidermal cells. Given that *MpGL3* has been used successfully for ISH on 2-week-old thalli and an ubiquitous activity of the *proMpGL3* has been shown, I used *MpGL3* as a technical control in the third attempt of ISH.

I synthesized new DIG-labelled probes for both *MpRSL1* and *MpGL3* and hydrolysed them to fragments of 100-250 bp, increasing the possibility for them to permeabilize the membrane, which should increase the chance of hybridization of probe and mRNA. I used 150 ng in the hybridization solution for both sense and antisense probes. Gemmae treated with *MpGL3* antisense were highly stained around the apical notches as

well as in RIs (Figure 4.3 F). The staining of MpGL3 sense treated gemmae showed a very similar staining pattern to the antisense treated gemmae (Figure 4.3 G). In both a strong signal can be observed around the apical notch and growing rhizoid cells, supporting the hypothesis that those tissues are more likely to get stained during an ISH treatment than other cells. Interpretations about the MpGL3 mRNA accumulation cannot be made due to the similar staining pattern in sense and antisense treated gemmae. Like MpGL3, colour development occurred in apical notch as well as in RIs in gemmae treated with MpRSL1 sense and antisense probes (Figure 4.3 L and M), which does not allow me to draw conclusive interpretations about the accumulation of MpRSL1 mRNA in 0-day-old gemmae. Both staining patterns, for MpGL3 and MpRSL1 transcript, support the hypothesis that meristematic cells and RIs are more likely to show a colour development than other tissue.

Summary of whole mount ISH

The staining pattern of antisense and sense treated samples are almost identical in all three attempts of whole mount ISH. The same cell types that are stained independent of the probe that was used are cells around the apical notch and RIs. Those results indicate that those cells either have a high activity of endogenous phosphatases that cause the colour development to be very strong covering the signal coming from the Antarctic Phosphatase activity (which is linked to the antibody bound to the DIG-labelled probes hybridized to the mRNA of interest). Alternatively, those are cells that are more likely to be permeabilized by probes compared to other epidermal cells, where they bind specifically and unspecifically to other target genes, causing a highly unspecific staining pattern.

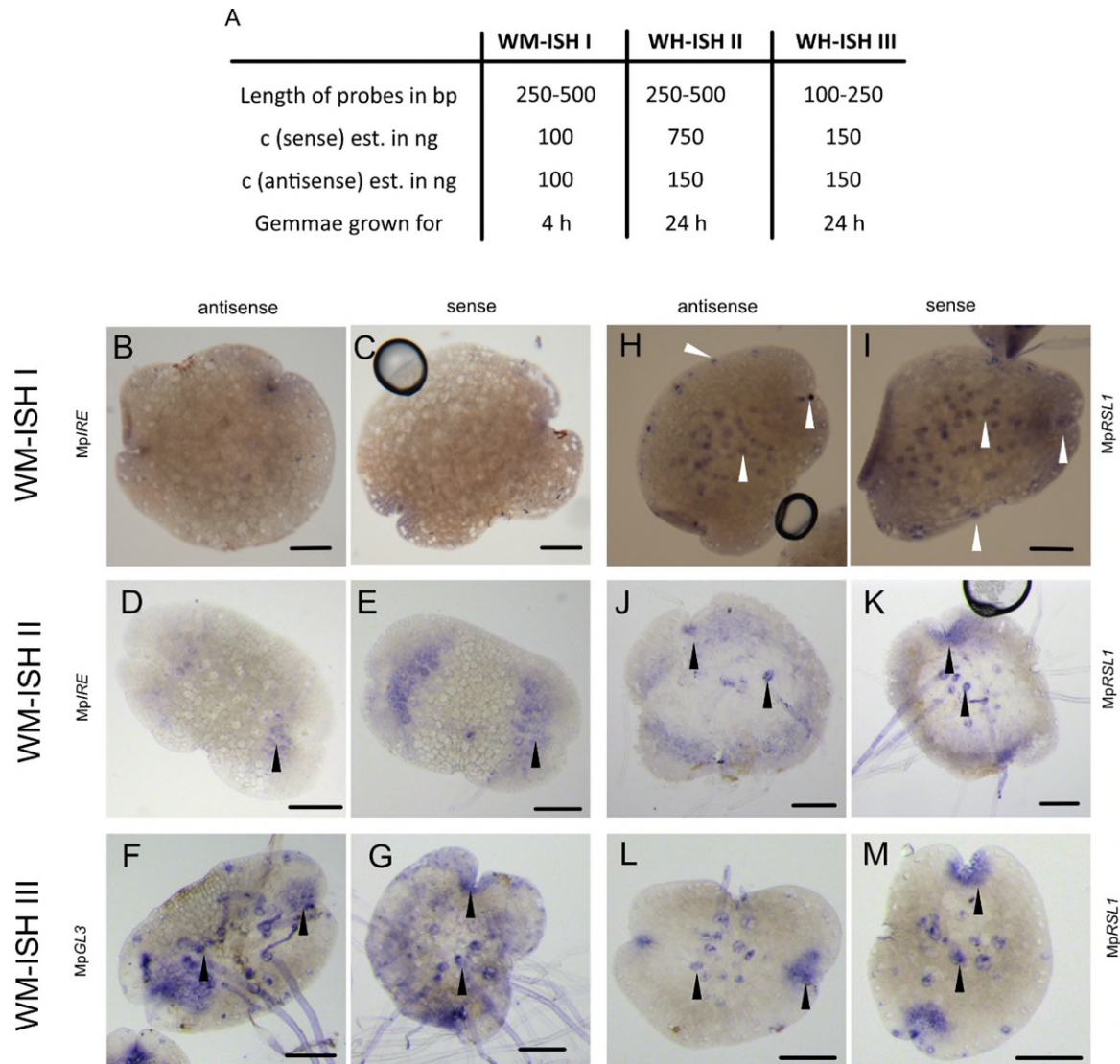


Figure 4. 6 Experimental conditions and outcome of three independent whole mount ISH on gemmae for *MpIRE*, *MpGL3* and *MpRSL1*

A) Length of antisense and sense probes in batch I (250-500 bp) in batch II (250-500 bp) and batch III (100-250 bp), concentration of sense and antisense probe used in all three batches. Last row indicates how many hours gemmae were grown before used for ISH.

Outcome of ISH-I for *MpIRE* antisense (B) and sense (C) and *MpRSL1* antisense (H) and sense (I). Outcome of ISH-II for *MpIRE* antisense (D) and sense (E) and *MpRSL1* antisense (J) and sense (K). Outcome of ISH-III for *MpGL3* antisense (F) and sense (G) and *MpRSL1* antisense (L) and sense (M). Scale bar 100µm

4.4 Discussion

Rhizoid formation in *M. polymorpha* is controlled by the interaction of the bHLH TF MpRSL1 mRNA and the miRNA MpFRH1. MpFRH1 controls the patterning of RIs by suppressing the development of rhizoid clusters in a mature gemmae (Chapter 3). The mature gemma is the product of numerous cell divisions, originating from an epidermal cell at the bottom of a gemma cup (see Introduction). During gemma development a disc shaped propagule develops with two apical cells opposing each other which, through active cell divisions, give rise to the two characteristic apical notches. It is important to note that the central part is most likely the product of cell divisions that happened early in gemma formation and hence the product from those cell divisions originates from the epidermal cell at the bottom of a gemma cup. Later on, when the gemma starts growing outside the gemma cup, newly developing RIs derive from the apical notch. Here I show that the promoter of *MpRSL1* is active in numerous cells around the apical notch and promoter activity of the *MpRSL1* becomes more restricted to RIs in the centre of mature gemmae. If we assume that the promoter activity reflects the pattern of *MpRSL1* transcription, it indicates that *MpRSL1* transcription is high around the apical notch and it is more restricted to RIs towards the centre of a gemma. *MpFRH1* promoter activity is high in mucilage papillae cells as well as in RIs. Activity of the mature miRNA MpFRH1 was detected in cells around the apical notch (section 4.3.1). The apical notch is the tissue where cell division, pattern formation and cell differentiation take place and all RIs that form after the gemma starts to grow will have its origin here. In the more central part of a mature gemma, no further cell differentiation (e.g. rhizoid differentiation) takes place and only cell elongation/ outgrowth occurs during further development. These findings suggest that *MpRSL1* is transcribed in apical notches, where MpFRH1 causes post transcriptional silencing of *MpRSL1* mRNA. MpFRH1 mediated post transcriptional silencing is likely to

reduce MpRSL1 expression to individual epidermal cells, which define which cells will differentiate, e.g. into RIs, generating the characteristic rhizoid patterning on *M. polymorpha* gemmae of individual well distributed RIs. I conclude that MpFRH1 miRNA is active in the regions where epidermal pattern formation is taking place.

The development of supernumerary RIs and the formation of RI clusters in *Mpfrh1^{LOF}* indicate that MpFRH1 acts as an inhibitor of ectopic RI formation and represses the development of connected specialised epidermal cells (chapter 3). The rhizoid defective phenotype in *Mpfrh1^{LOF}* affects RIs in the centre of a mature gemma, while MpRSL1 promoter activity and MpFRH1 activity are observed in the apical notch. It is important to note that RIs in the centre of a gemma are likely not to be the product of cell divisions and differentiation originating in the apical notches. However, I hypothesise that MpFRH1 affects the newly developing RIs coming from the apical notch in the same way than it does with the RIs in the central region of a mature gemma. To test that hypothesis it would be important to follow the development of RIs in *Mpfrh1^{LOF}* originating from the apical notch to see if rhizoid clusters form as well.

Interestingly, the promoter of *MpFRH1* is active in RIs, which suggests that MpFRH1 acts as a mobile inhibitor of RI formation, hence it acts non-cell autonomously. The presence of an inhibitor in cell types that they are suppressing is similar to the reaction diffusion model initially described by Alan Turing (Turing, 1952). This model describes how two interacting chemicals – an activator and an inhibitor - that are diffusing through space can form interacting wave patterns to produce individual peaks of activating signal, which is translated into individual spots (Figure 4.7A, B). The pre-pattern of a Turing pattern consists of a spatially uniform steady state of the two morphogens (Figure 4.7B), activator and inhibitor. However, small fluctuations or noise within the equilibrium can cause the emergence of small local maxima of the activator (Figure 4.7B). The activator

and inhibitor are diffusing signals, while the inhibitor diffuses faster than the activator. The activator amplifies its own synthesis through self-activation as well as inducing the production of its own inhibitor. This causes the small maxima to increase and to become even more distinct and through lateral inhibition (mobile inhibitor), no other maxima can raise and sharp boundaries of activator expressing, and non-expressing cells develop (Figure 4.7 A,B, Meinhardt and Gierer, 2000).

This model has been used to explain the formation of trichomes in *A. thaliana* (Torii, 2012) which, as already discussed in chapter 3, shows a very similar patterning formation to the RI pattern on the epidermal surface of *M. polymorpha* gemmae. While MpRSL1 constitutes an activator of rhizoid determination, MpFRH1 is an inhibitor of MpRSL1 and hence of rhizoid determination. If we apply the Turing model to rhizoid patterning in *M. polymorpha* we would assume that activator (MpRSL1) and inhibitor (MpFRH1) are uniformly present before cell differentiation takes place. Subsequently, local MpRSL1 maxima would rise, which would become amplified due to a self-activating characteristic of the activator (was not tested in this thesis). The activator causes the activation of an inhibitor, here MpFRH1 at their local maxima sites. The inhibitor would then act as a mobile signal and diffuses or becomes actively transported into neighbouring epidermal cells, causing lateral inhibition around emerging rhizoid cells (Figure 4.7 C).

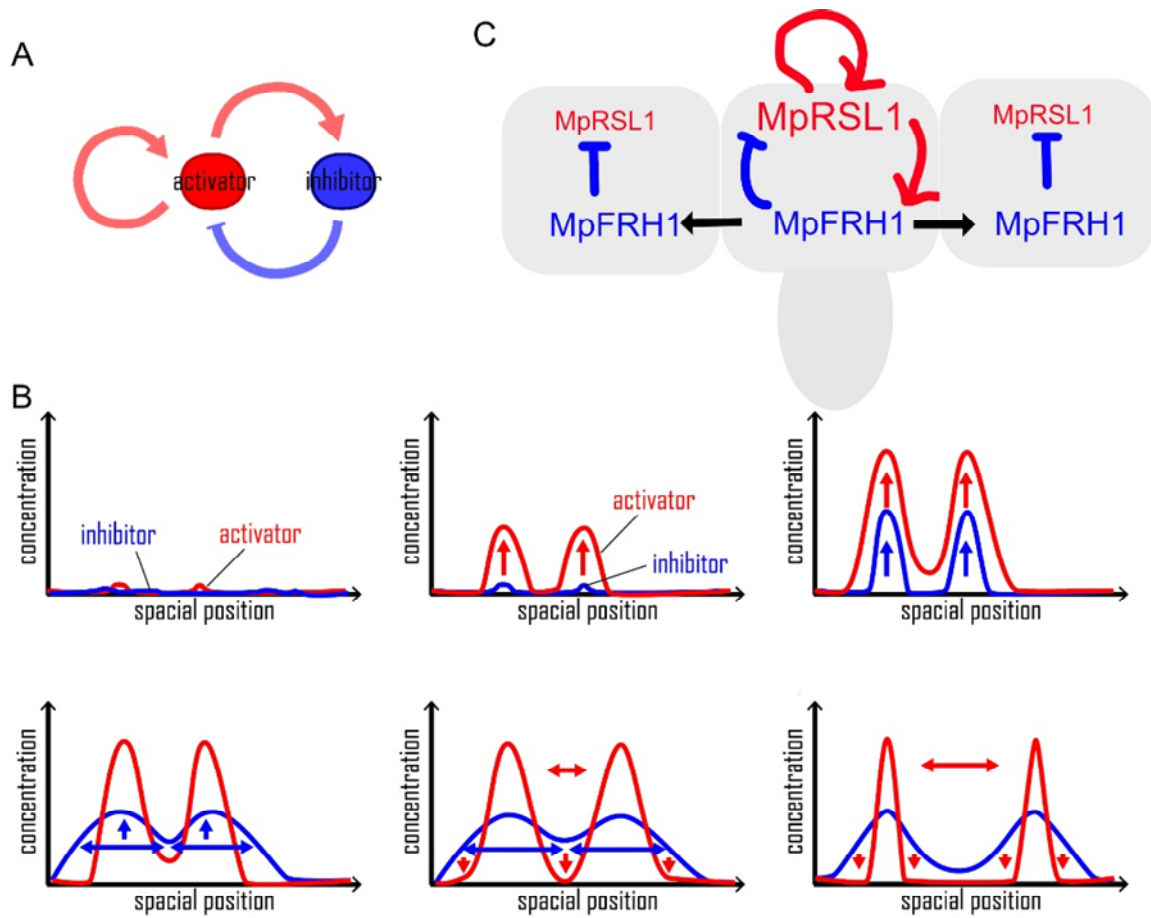


Figure 4. 7 Turing model and proposed mechanism of *MpRSL1*-*MpFRH1* interaction during rhizoid formation

A) Interaction of activator and inhibitor associated with Turing model: activator with self-activating property, which activates its own inhibitor B) generation of a periodical pattern according to Turing: initially there is no preformed pattern, concentration of activator and inhibitor are homogenous throughout the field. Small fluctuations of activator/inhibitor concentrations within the field caused by perturbations lead to small peaks of activator. Concentration of activator becomes higher and higher (due to self-activating property) as well as concentration of inhibitor (due to induction through activator). Inhibitor diffuses faster than activator, causing the concentration of inhibitor to become relatively high around that point and that of activator becomes low due to inhibition = lateral inhibition. Eventually a spot of activator is generated. C) Proposed model of *MpRSL1* and *MpFRH1* and their interaction during rhizoid formation. *MpRSL1* (= activator) maximum in individual rhizoid cells is generated due to self-activation of activator as well as activation of *MpFRH1* (= inhibitor). *MpFRH1* moves to adjacent cells, reducing *MpRSL1* expression (= lateral inhibition), which restricts *MpRSL1* accumulation and rhizoid formation to individual epidermal cells.

<http://2015.igem.org/Team:UT-Tokyo/Project>

The data presented here indicate that indeed *MpRSL1* is transcribed uniformly in the apical notch, where cell division takes place and cell differentiation is set up. The promoter of *MpFRH1* is active in mucilage papillae cells, cells surrounding the apical cell. Hence, activator and inhibitor are both present in tissues where cell patterning is initiated. I showed that *MpFRH1* is active in the apical notch, indicating that *MpRSL1*-*MpFRH1* interaction occurs in this region of the gemma. If *MpFRH1* and *MpRSL1* follow the

characteristic of Turing morphogens, MpRSL1 maxima rise due to self-activation of MpRSL1 but also due to the activation of MpFRH1 that acts non-cell autonomously to mediate post transcriptional silencing of MpRSL1 in cells surrounding the developing maxima. Due to this activity, sharp maxima of activator rise. Since the promoter of MpRSL1 is broadly active around the meristematic notch and its activity becomes more restricted towards the central gemmae, it suggests that MpRSL1 transcription is unspecific in undifferentiated tissue but becomes more specific in differentiated tissue. This expression pattern suggest that during rhizoid formation the transcription of MpRSL1 becomes restricted to individual cells that differentiate into rhizoid cells, causing the establishment of very defined MpRSL1 maxima, which is consistent with Turing reaction-diffusion activity. If MpFRH1 acts indeed as a mobile inhibitor causing lateral inhibition of MpRSL1, no lateral inhibition of MpRSL1 mRNA would occur in *Mpfrh1^{LOF}*. Indeed, the development of RI clusters in *Mpfrh1^{LOF}* suggest that no lateral inhibition takes place, which supports the idea of MpFRH1 acting as a mobile inhibitor. A possibility to test the mobility of MpFRH1 is the expression of the viral repressor p19 in *M. polymorpha* wildtype plants. Expression of p19 results in 21nt small RNA duplexes being sequestered into a cell autonomous complex - preventing mature miRNAs from moving to neighbouring cells. If MpFRH1 acts as a mobile miRNA moving from developing RIs into neighbouring epidermal cells, wildtype plants expressing the p19 repressor would show an *MpFrh1⁻* phenotype, indicating that MpFRH1 indeed generates an inhibitory field radiating from developing RIs. Additionally, miRNAs in Arabidopsis move through plasmodesmata (Vatén et al., 2011). Through the increased deposition of callose those plasmodesmata can be narrowed so that passage of molecules is highly reduced (Coudert et al., 2015). If MpFRH1 passes plasmodesmata to establish lateral inhibition, the closure of plasmodesmata reduces its ability to move and the formation of rhizoid cluster would occur

in wildtype. This could be tested by overexpressing a callose synthase (e.g. an orthologue of the Arabidopsis *CALLOSE SYNTHASE 3*), causing the closing of plasmodesmata in *M. polymorpha* (Vatén et al., 2011; Coudert et al., 2015).

If this model operates in *M. polymorpha*, MpRSL1 should positively regulate its own expression. This can be tested experimentally by chromatin immunoprecipitation (ChIP), which can identify DNA sequences that interact with MpRSL1 protein. This way I could test if MpRSL1 directly activates its own transcription. At the same time, I could identify other putative downstream targets of MpRSL1 and show whether MpRSL1 also positively regulates MpFRH1 abundance. Hence, another possible direct target of MpRSL1 is the promoter of MpFRH1, which could be identified in a ChIP experiment.

Taken together, the findings I presented in this chapter significantly increase our understanding of how MpRSL1 and MpFRH1 interaction controls the patterning formation of rhizoids in *M. polymorpha*. Based on these results I propose that MpFRH1 and MpRSL1 represent two morphogens controlling rhizoid patterning. These early insights open up the possibility that MpFRH1 and MpRSL1 follow the criteria of inhibitor and activator based on the reaction diffusion model described by Alan Turing. This hypothesis helps to explain how complex cell types develop and patterning occurs in *M. polymorpha*.

4.5 Material and Methods

4.5.1 Plant material

M. polymorpha accessions Tagaragaike-1 (Tak-1, male) and Tagaragaike-2 (Tak-2, female) were used as wildtype (Ishizaki et al., 2008). Plants were grown on sterile plates containing Johnson's medium (see chapter 3.4.8). Plants were growing under continuous white light illumination ($56 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) at 23°. Gemmae of mature plants were collected for further analysis.

4.5.2 Generation of MpRSL1 promoter reporter line and MpFRH1 sensitive/resistant reporter lines

The 5kb long promoter sequence of MpRSL1 was amplified using the primer oAT123 and oAT41 and the Phusion High-Fidelity DNA Polymerase (ThermoFisher) following manufacturer's instruction. This PCR fragment was purified following manufactures instructions (QIAquick Gel Extraction Kit). By performing a second PCR using the purified fragment as template, 15 bp overhangs complementary to pCAMBIA sequence upstream and downstream of EcoRI restriction site as well as a HpaI restriction site at the reverse primer were added, using oAT163 and oAT164. The promoter sequence including HpaI restriction site was inserted into EcoRI digested pCAMBIA using in-fusion cloning kit following manufacturers instructions (In-Fusion cloning, Clontech). As a result, the MpRSL1 promoter was inserted into the MCS (at the EcoRI position) downstream of the 35S promoter, facing the same direction than the 35S promoter. In the next step this vector was HpaI digested, and a Gateway cassette (fragment A), was inserted following manufacturer instructions (Thermo Fisher). In the following 3xNLS-YFP was inserted into proMpRSL1:GW:pCAMBIA performing a LR reaction using pHB39 as entry vector

following manufacturer's instructions (Thermo Fisher). The final expression vector *proMpRSL1:3xNLS-YFP* was transformed into *Agrobacterium tumefaciens*. Following, *M. polymorpha* spores were transformed following the protocol described in chapter 3.4.3.

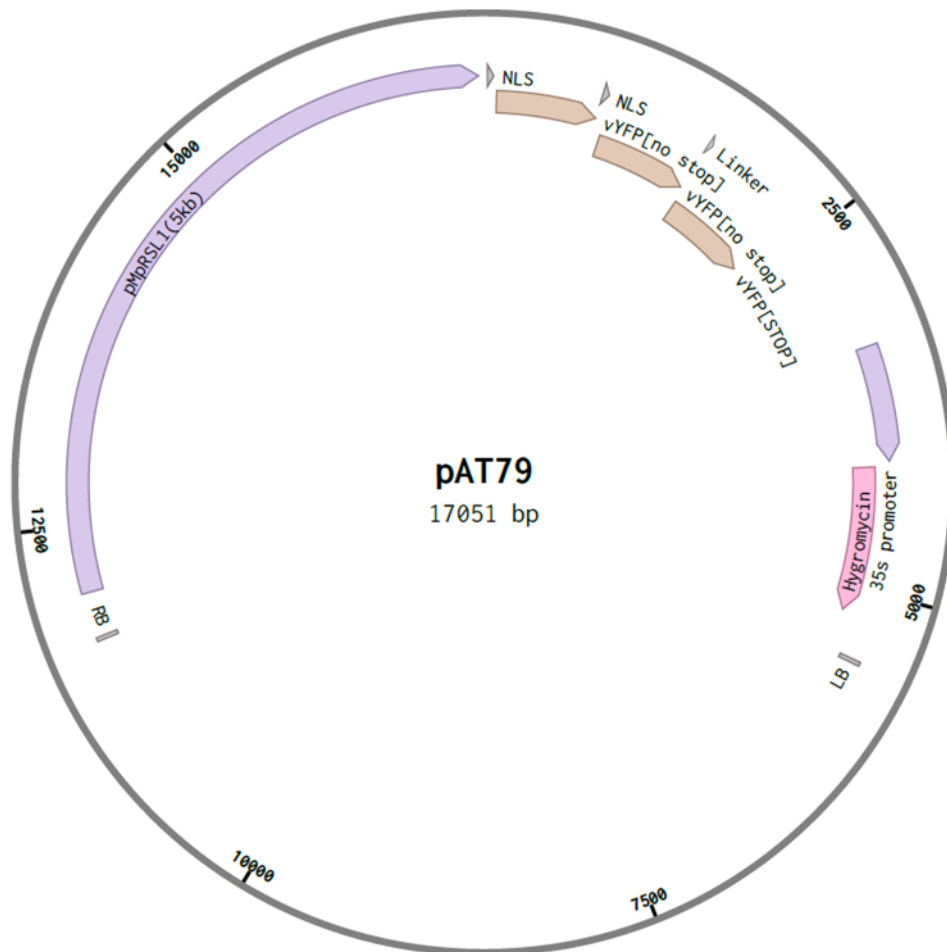


Figure 4. 8 Vector map of *proMpRSL1:3xNLS-YFP*

proMpRSL1 (5kb) drives the expression of three *NLS-YFPs*, the backbone also harbours a hygromycin resistance gene under a 35S promoter (purple)

4.5.3 Laser Confocal Imaging

Images were acquired with a Leica SP5 laser confocal microscope and the Leica Application Suite (LAS) software using either a Leica HCX PL Fluotar 10x/0.30 or HC PL APO 20x/0.75 IMM CORR CS2 lense. Chlorophyll was excited at 543 nm and emission was collected between 680-700nm for chlorophyll. YFP and CFP fluorescence was excited at 514 nm using an argon laser and emission was measured between 524 and 561 nm (YFP) and between 463 and 561 nm (CFP) using an Acousto Optical Tunable Filter.

4.5.3 Image analysis using Fiji

Image analyses and plant/cell measurements were performed in ImageJ. First, I quantified the maximum pixel value of the image. For further quantification I selected images that do not exceed a maximum pixel value of 255 or if only individual pixel were saturated. The maximal pixel value was used to quantify the relative pixel intensity of YFP signal of interest. I selected 25 to 30 nuclei surrounding the apical notch and measured their brightest YFP signal using the multi point tool in ImageJ (see figure 4.3 B). I then calculated the average YFP of those cells in at least 5 biological replicates.

4.5.4 ISH on sections

ISH on sections followed the basic protocol of Jackson (1991) and Coen et al. (1990), with modifications which are summarized in the following paragraphs.

Tissue preparation and sectioning

Gemmae were grown for 6 hours and fixed in a 1:1 mixture fixative (containing 4 % paraformaldehyde, 15 % DMSO and 0.1 % Tween- 20):heptane for 45 min on a rotary shaker at room temperature, following treatment with 100% methanol (2 times 5 minutes)

and 100% ethanol (3 times 5 minutes). Samples were stored in 100% ethanol at -20°C upon use. Gemmae were embedded into 2% agar. Agar blocks of about 0.5cmx0.3cm were cut out, placed into embedding cassettes and incubated for at least 2 hours with 100% Ethanol. Samples were embedded in wax using Histo-Tek® VP1™ (Sakura) with the following incubation steps: 3x 100%EtOH for 60' at 35°C, 3x 100% HistoClear for 1 hour at 35 °C, 2x wax for 1hour at 62°C, 2x wax for 2 hours at 62 °C. Agar blocks were embedded in paraffin using the Tissue-Tek TEC5 Tissue Embedding Console System (Sakura) following sectioning of agar blocks into 8 µm thick sections.

Probe preparation

RNA probes were synthesised using SP06 and T7 polymerase and hydrolysed into 100-250 bp long molecules following Hejártko et al. (2006). Probe concentration was determined using dot blot analysis as described in Brewer et al. (2006).

ISH

Each slide with plant sections was pre-treated following the previously mentioned protocols, including Proteinase K treatment (125 µg/ml, for 4' at 37°C) and Glycine treatment (0.2% in PBS). Sections were refixed in 4% formaldehyde in PBS. Unspecific binding of probes was reduced by an acetic anhydride treatment (1ml acetic anhydride in 0.1M Triethanolamine, pH 8.0). Each slide was emerged with 2.5µl probe (antisense or sense, e.g. 25ng of DIG labelled RNA), 5.5 µl water and 8 µl formamide and 64µl of hybridization buffer (1.25x Salts, 50% formamide, 12.5% Dextranulphate, 0.3125-2.5mg/ml tRNA, 1.25% Denhart's solution in water). Slides were covered with sterile RNase free cover slips, and incubated in 2x SSC,50% Formamide. Slides were incubated at 50 °C for at least 18hours. Afterwards the slides were washed several times with NTE. RNase treatment (20 µg/ml) digests any unspecifically bound single stranded RNA without affecting the specifically bound, hybridized double stranded probe-RNA. DIG labelled

probes were labelled with anti-DIG antibody coupled with alkaline phosphatase (Boehringer) in a concentration of 1:3000 for 1 hour at RT and if desired overnight at 4 °C. Colour development was initiated by incubating the slides with commercial NBT/BCIP substrate solution (Thermo Fisher). Colour detection was stopped when signal in sense treated samples occurred, or after maximum 5 days of staining.

Chapter 5

General discussion

MiRNA activity during development is indispensable for controlled cell divisions and cell differentiation in plants. I characterised the interaction of the miRNA MpFRH1 and its direct target MpRSL1 and how their interaction controls the differentiation of specialised epidermal cells, in particular of rhizoid cells. The development of other epidermal cells into specialised structures is also controlled by MpFRH1 and MpRSL1, e.g. mucilage papillae cells as well as the formation of meristematic notches.

MpRSL1 is a bHLH TF and the only member of RSL class I genes in *M. polymorpha*. In contrast, *A. thaliana* has two members of this gene family, *AtRHD6* and *AtRSL1*. The function of RSL class I TFs is conserved among land plants since they control rhizoid development in bryophytes, e.g. *P. patens* and *M. polymorpha* as well as root hair formation in angiosperms, e.g. *Brachypodium distachyon* and *A. thaliana* (Kim and Dolan, 2016). While RSL class I genes in *A. thaliana* are negatively controlled by GLABRA2 (GL2), this thesis shows that the RSL class I gene in *M. polymorpha* is controlled by the miRNA MpFRH1. These findings demonstrate the independent evolution of negative regulatory mechanisms of an evolutionary conserved regulator. This is interesting as it constitutes an example of how conserved genetic mechanisms can be controlled (upstream) in different ways and that miRNAs are important regulators in controlling these mechanisms.

Most of the miRNAs found in bryophytes are lineage-specific. MpFRH1 miRNA is no exception as it is liverwort-specific. The abundance of lineage-specific miRNAs highlights the rapid evolution of miRNAs that carry out unique (lineage-specific) and very specific functions. Hence, they could contribute to the introduction of novel function, given their rapid evolution and the great diversity of plant morphology. The functional characterization of conserved and non-conserved miRNAs in diverse organisms will reveal the evolution of miRNAs in various lineages – when they evolved, when they were lost,

how their interaction with target genes changes and how this interaction controls developmental processes. MpFRH1 is a miRNA that only evolved in the liverwort lineage, after liverworts diverged from the other extant lineages of land plants. No other miRNA, in particular no MpFRH1-like miRNA, has been found that controls RSL class I expression in other land plant lineages.

Here I show that MpFRH1 is a miRNA controlling rhizoid patterning, by suppressing the development of RI clusters in mature gemmae. Similar changes in the formation of mucilage papilla cells and meristematic notches suggest a common underlying mechanism, by restricting the differentiation of epidermal cells into specialised structures to individual cells only. Molecular evidence suggests that MpFRH1 acts non-cell autonomously by radiating from developing RIs around the apical notch into neighbouring epidermal cells to repress MpRSL1 and hence rhizoid formation. If this hypothesis were correct, MpFRH1 would transmit positional information through lateral inhibition. This would represent a new mechanism by which miRNAs control cell differentiation by radiating in a single cell layer. As discussed in chapter 4, the phenotypic defects observed in *Mpfrh1^{LOF}* relate to RIs that originated during gemma formation, most likely through cell divisions and differentiations originating from the gemma initial cell, an epidermal cell at the bottom of a gemma cup. MpFRH1 activity and the MpRSL1 promoter activity was also detected in the apical notch, which will give rise to new tissue, including new RIs. To test if both RIs deriving from the apical notch and RIs deriving from the gemma initial are controlled by MpFRH1-MpRSL1 activity, it is crucial to study the development of RIs deriving from the apical notch on *Mpfrh1^{LOF}*.

If MpFRH1 acts non cell autonomous to suppress MpRSL1 in adjacent epidermal cells, this interaction could represent one of very few biological examples where two morphogens have been identified that could establish pattern formation following the

Turing patterning mechanism. A patterning mechanism that is partially understood is the spacing of trichomes in *A. thaliana* leaves. Even though the formation follows a Turing patterning mechanism, it is much more complex. Here, the bHLH/WD40/MYB complex activates GL2, which is a positive regulator of trichome formation. TRY, CPC or ETC2 then act as mobile signals radiating from developing trichome cells, where it interacts with the bHLH/WD/MYB complex in neighbouring epidermal cells. The activation of GL2 is suppressed and no trichome development occurs in adjacent cells. If MpFRH1 indeed radiates as a MpRSL1 suppressing signal into neighbouring cells from developing rhizoid cells, the establishment of trichome and rhizoid patterning followed a very similar mechanism, conducted by different molecular regulators. A lateral inhibition mechanism underlying rhizoid formation would be interesting as it would demonstrate an example how similar patterning mechanisms evolved independently by recruiting a different set of regulatory genes, e.g. the mediating suppressor signal in rhizoid patterning is a miRNA while in trichome formation the mediating suppressor signal is a R3 Myb protein.

The fact that MpFRH1 and MpRSL1 might function as Turing morphogens during epidermal patterning in *M. polymorpha* could allow us to study the Turing model in a new biological system which is relatively easy to manipulate. By changing the properties of MpFRH1 or MpRSL1 it would be possible to study the effects of activator and inhibitor during patterning establishment.

Furthermore, if MpFRH1 can be confirmed to act non-cell autonomously, this system constitutes a powerful system to study a still open question in the miRNA field. Much evidence supports the generally-accepted hypothesis that miRNAs move via plasmodesmata from cell to cell (Chitwood and Timmermans, 2010; Vatén et al., 2011; Pyott and Molnar, 2015). The hypothesis that miRNAs act non-cell autonomously is mainly based on experiments showing that miRNAs accumulation is detected in cells next to cells

where the miRNA is processed (Carlsbecker et al., 2010; Skopelitis et al., 2017) and miRNA activity to be present in cells neighbouring cells where miRNAs are produced (Pyott and Molnar, 2015; Skopelitis et al., 2017). However, methods are not currently available to label miRNAs in vivo to show their ability to move from cells to cells. To demonstrate that MpFRH1 acts non-cell autonomously one experimental approach would be to generate *Mpfrh1^{LOF}*/wildtype chimeric plants (e.g. inducing *Mpfrh1* mutations through CRISPR/Cas9 after induction). If the *MpFrh1^{LOF}* phenotype can be suppressed in genetically *Mpfrh1^{LOF}* tissue close to genetically wildtype tissue, they must be caused by the mobile properties of the miRNA MpFRH1. This would support the hypothesis that miRNAs like MpFRH1 act as moving signals. Moreover it would provide information on how far miRNAs can move.

Furthermore the *M. polymorpha* serves as a system to prove the mobility of MpFRH1 – assuming that MpFRH1 passes plasmodesmata to reach its neighbouring cells – by inducing the closure of plasmodesmata (Bilska and Sowiński, 2010; Coudert et al., 2015). When plasmodesmata are closed, miRNA emigration is reduced and the formation of rhizoid cluster would occur in wildtype.

Alternatively, miRNA movement could be effected by an active mechanism, allowing some miRNAs to be transported to neighbouring cells while others do not exit their cells of origin and act cell autonomously. If an active transport underlies miRNA movement, *M. polymorpha* presents a good system to screen for mutants that resemble the defective phenotype of *Mpfrh1^{LOF}* mutant gemmae. Those mutant plants are likely to have a mutation in the MpFRH1 movement pathway. Some of the genes are likely to regulate miRNA synthesis and processing but also orchestrate MpFRH1 cell-cell movement.

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