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Efficient and Inducible Use of Artificial MicroRNAs in *Marchantia polymorpha*

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We describe the efficient use of artificial microRNAs (amiRs) in *Marchantia polymorpha* using both endogenous and heterologous primary microRNA (pri-miR) hairpin backbones. Targeting of two transcription factor genes, *MpARF1* and *MpRR-B*, mediating different hormonal responses, demonstrated that amiRs can create specific and reproducible physiological and morphological defects, facilitating interpretation of gene function. A third amiR was designed to target a gene encoding a component of the Polycomb recessive complex 2, *MpE(z)*, and constitutive expression of this amiR results in sporeling lethality. Adaptation of an estrogen-inducible system allowed analysis of the phenotypic effects of induction of this amiR during other stages of the life cycle. We discuss the advantages and challenges of the use of amiRs as a tool for reverse genetic analysis in *M. polymorpha*.

Keywords: Artificial microRNA • AUXIN RESPONSE FACTOR • ENHANCER OF ZESTE • *Marchantia* • MicroRNA • RESPONSE REGULATOR • Reverse genetics.

Abbreviations: AGO, ARGONAUTE; amiR. artificial microRNA; ARF, AUXIN RESPONSE FACTOR; BA, 6-benzylaminopurine; CRISPR, clustered regularly interspaced short palindromic repeats; EF1-α, elongation factor 1-α; E(z), ENHANCER OF ZESTE; G418, geneticin; Hyg, hygromycin; miR, micro RNA; MIR, primary microRNA loci; pre-miR, precursor microRNA; pri-miR, primary microRNA; PRC2, Polycomb repressive complex 2; RISC, RNA-induced silencing complex; RLM 5′-RACE, RNA ligase-mediated 5′ rapid amplification of cDNA ends; RR, RESPONSE REGULATOR; RT–PCR, reverse transcription–PCR; TCP, TEOSINTE BRANCHED/CYCLOIDEA/PCF; XVE, lexA DNA-binding domain/VP16-transactivating domain/human estrogen receptor.

Introduction

In plants, microRNAs (miRs) are a class of small silencing RNA encoded by a primary miRNA (pri-miR) transcript that are transcribed by RNA polymerase II and subject to distinct spatio-temporal expression patterns (Reinhart et al. 2002, Dugas and Bartel, 2004, Jones-Rhoades et al. 2006). DICER-LIKE endoribonucleases recognize pri-miR transcripts, and in a two-step process create a precursor-miR hairpin (pre-miR) that is subsequently cleaved to create a 21 nucleotide miR/miR* duplex. Although one of the strands of this duplex (the miR) is preferentially loaded into the RNA-induced silencing complex (RISC) that contains catalytic AGO (ARGONAUTE) proteins, more recent studies have shown that miR* strands can also be loaded with AGO proteins to direct specific suppression of target gene transcripts (Ossowski et al. 2008, Bartel 2009, Zhang et al. 2011, Jeong et al. 2013, Coruh et al. 2014). Preferential loading of a mature miR into RISC depends on differential 5′ (miRs almost always start with U) and 3′ stability (high GC content in position 17–19). Pri-miR transcripts can be several kilobases in length and the mature miR can be derived from the 5′ or 3′ arm of the pre-miR hairpin. In multiple plant species, ectopic overexpression of the basic pri-miR hairpin has proven sufficient to create loss-of-function knockdowns (Aukerman and Sakai 2003, Palatnik et al. 2003, Chen 2004, Alvarez et al. 2006, Schwab et al. 2006)

Artificial substitution of the 21 nucleotide miR and miR* sequence of an endogenous miR precursor to a different target specificity creates an artificial miR (amiR) and has proven a feasible way to down-regulate genes of interest for developmental and physiological studies (Alvarez et al. 2006, Schwab et al. 2006, Ossowski et al. 2008). The use of amiRs has been implemented in species in several lineages of the Viridiplantae to study gene function, including the unicellular Chlorophyte alga *Chlamydomonas reinhardtii* (Molnar et al. 2009), the moss *Physcomitrella patens* (Khraiwesh et al. 2008) and a diverse range of angiosperm species including monocots (Warthmann et al. 2008) and several eudicots (Alvarez et al. 2006, Schwab et al. 2006).

Artificial miRs, as with others RNA polymerase II transcripts, are subject to inducible expression. Reliable inducible expression of knockdown constructs is a powerful tool in genetic analysis, particularly if null phenotypes are potentially lethal. Several inducible systems have been developed for use in transgenic plants, all with their own advantages and disadvantages. For example, ethanol induction (Deveaux et al. 2003) has its limitation from the volatility of the inducer that can activate neighboring plants (Roslan et al. 2001); heat shock (Saidi et al. 2005) and Cre/lox systems (Wachsman and Heidstra, 2010) can stress plants and are either only active for a short time (heat shock), or once switched on cannot be reversed (Cre/lox).

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In this study. we explore the use of amiRs in the liverwort *Marchantia polymorpha*, a member of the basal-most lineage of land plants. Previous studies in *M. polymorpha* have developed tools to create deletion alleles using homologous recombination (Ishizaki et al. 2013) or CRISPR-CAS- (clustered regularly interspaced short palindromic repeats) mediated genome modification (Sugano et al. 2014). These two techniques create primarily null loss-of-function alleles and, thus, have limitations when such alleles result in lethality. To overcome these limitations, which are particularly acute in a haploid organism, techniques to create conditional or inducible alleles are required. We demonstrate that the design of amiRs in *M. polymorpha* provides an alternative tool to study gene function in a promising emerging genetic model system.

Results

Artificial microRNA design in *Marchantia*

In this study we designed amiRs targeting three loci in *M. polymorpha*: the class A *AUXIN RESPONSE FACTOR1* (*MpARF1*) involved in auxin signaling for which the null loss-of-function phenotype is known; the single ortholog of the type-B *ARABIDOPSIS RESPONSE REGULATOR* (*MpRR-B*) family of transcription factors with previously uncharacterized phenotypes in *M. polymorpha*; and the ortholog of *ENHANCER OF ZESTE* [*MpE(z)*], which encodes a component of the Polycomb repressive complex 2 (PRC2). Multiple loss-of-function mutants of *E(z)* orthologs in *Arabidopsis thaliana* (*SWINGER* and *CURLY LEAF* double mutants) grow as undifferentiated embryo-like cells, with a triple *MEDEA*, *SWINGER*, *CURLY LEAF* mutant possibly lethal (Chanvivattana et al. 2004). Thus, we surmised that *MpE(z)* might be amenable for study only using an inducible system for amiR expression (**Fig. 1A**).

In *A. thaliana*, *MIR319* is often used as a backbone for amiR construction. Although there are two putative *miR319* loci in *M. polymorpha*, neither of the two *M. polymorpha* TCP (TEOSINTE BRANCHED/CYCLOIDEA/PCF) family members, orthologs of which are *miR319* targets in *A. thaliana* (Axtell et al. 2007), have a conserved miR319 target site, complicating the corroboration of activity of *MpmiR319* in *M. polymorpha*. Thus, we examined the activity of two other microRNAs conserved across land plants, *miR160* and *miR166*. A 221 bp fragment spanning the stem–loop structure of *MIR160a* derived from and previously shown to be active in *A. thaliana* was inactive in *M. polymorpha* (**Supplementary Fig. S1**). In contrast, 246 bp spanning the endogenous *MpmiR160* produces a stable phenotype, which will be described in detail in further studies (**Supplementary Fig. S1**). *miR166* has been shown to be active in all land plants examined (Floyd and Bowman 2004). Thus, we chose to use *MpMIR160* and a fragment of 246 bp spanning *SkMIR166*, derived from *Selaginella kraussiana*, as backbones to design amiRs for use in *M. polymorpha*. Using the *MpMIR160* precursor, *amiR-MpARF1*^{MpMIR160}, *amiR-MpRR-B*^{MpMIR160} and *amiR-MpE(z)*^{MpMIR160} were constructed, and a second version of *amiR-MpE(z)*^{SkMIR166} was designed using *SkMIR166*.

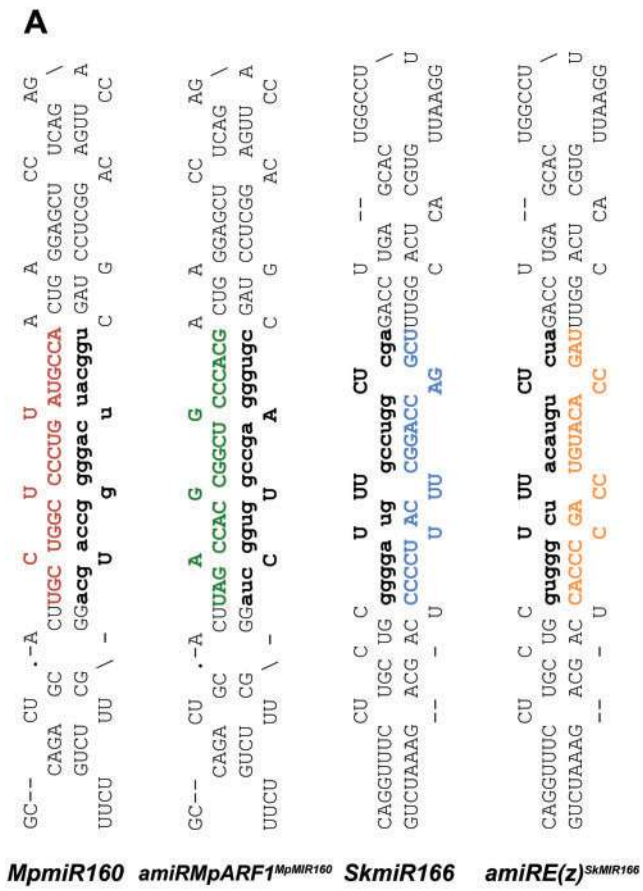
Vectors designed to express amiRs in *Marchantia*

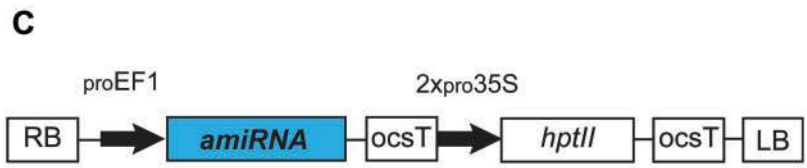
To create potentially strong knock-down phenotypes, *amiR* genes were expressed under the control of the elongation factor 1-α (EF1-α) regulatory sequences ($_{pro}$EF1) derived from the endogenous *MpEF1* gene encoding a translation elongation factor. These regulatory sequences drive high level constitutive expression (Althoff et al. 2013). The amiRs were either driven in *cis* directly downstream of the $_{pro}$EF1 regulatory sequences ($_{pro}$*EF1:amiR*; **Fig. 1C**) or in *trans* (*proEF1>>amiR*), with $_{pro}$EF1 driving expression of the estrogen-responsive chimeric XVE transcription factor (Zuo et al. 2000, Brand et al. 2006). In the presence of β-estradiol, XVE translocates from the cytoplasm to the nucleus, binding to lexA operator sequences, which in turn drive the amiR of interest (**Fig. 1D**). All the amiR constructs were inserted into a binary T-DNA vector conferring hygromycin (Hyg)/geneticin (G418) resistance in planta based on pMLBART (Gleave 1992). A T-DNA conferring only antibiotic resistance in planta was used as a control in simultaneous transformation experiments (**Fig. 1E**).

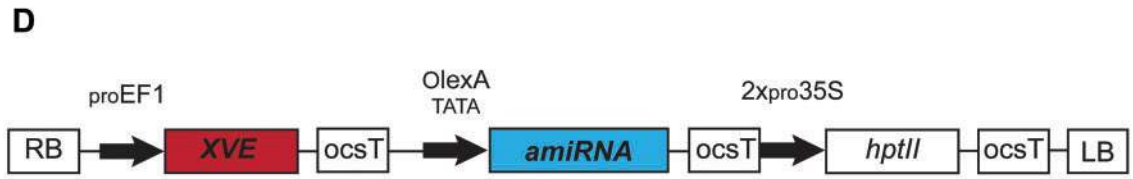
amiRs produce novel loss-of-function phenotypes

Arabidopsis type-B RESPONSE REGULATORS (RR-Bs) are involved in establishing positive transcriptional outputs in response to cytokinin as part of a multistep phosphorelay system in *A. thaliana* (Mason et al. 2005, Yokoyama et al. 2007, Argyros et al. 2008, Ishida et al. 2008). The *M. polymorpha* genome encodes a single RR-B (*MpRR-B*) as compared with 11 paralogs found in *A. thaliana*. We designed an amiR targeting *MpRR-B* using the *MpMIR160* precursor as a backbone and drove its expression with the EF1 promoter ($_{pro}$*EF1:amiR-MpRR-B*^{MpMIR160}). In a subpopulation of primary transformants, 63 out of 96 $_{pro}$*EF1:amiR-MpRR-B*^{MpMIR160} lines displayed ectopic serrations in thallus margins (**Fig. 2A, B**; **Table 1**), lacked gemmae cup formation (**Fig. 2C**) and were smaller in size after 40 d of growth (**Fig. 2D, E**; **Supplementary Fig. S2**A). To ascertain whether the phenotype observed in $_{pro}$*EF1:amiR-MpRR-B*^{MpMIR160} lines reflects a loss of cytokinin-mediated gene expression, primary transformants were plated in 40 µM 6-benzylaminopurine (BA) to assess resistance to high levels of exogenous cytokinin. In response to exogenous cytokinin, control plants grown on plates supplemented with 40 µM BA exhibit a reduction in branching frequency, with a 35% decline in the number of notches (representing sites of shoot apical meristems) and, in addition, a 44% decline in gemmae cup production. Compared with control lines, $_{pro}$*EF1:amiR-MpRR-B*^{MpMIR160} lines exhibit only 16% and 9% decreases in notch and cup production, respectively, indicating that knockdown $_{pro}$*EF1:amiR-MpRR-B*^{MpMIR160} lines are less sensitive to the effects of exogenous cytokinin (**Supplementary Fig. S2**B, C).

We detected the predicted cleavage products of *MpRR-B* in $_{pro}$*EF1:amiR-MpRR-B*^{MpMIR160} transformants (**Fig. 2F**) via RNA ligase-mediated 5′ rapid amplification of cDNA ends (RLM 5′-RACE) (Llave et al. 2002, Kasschau et al. 2003). In line with previous studies, the amiRs direct cleavage of targets between bases 10 and 11 of the miR-binding site (Lingel et al. 2003,







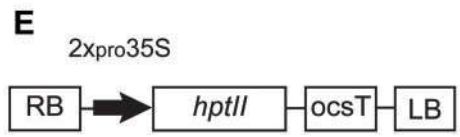


Fig. 1 Artificial microRNA design and expression strategy. (A) Predicted stem–loops formed by *MpmiR160*, *amiR-MpARF1*^{MpMIR160}, *SkmiR166* and *amiR-MpE(z)*^{SkMIR166}. *amiR-MpARF1*^{MpMIR160} retains the predicted stem–loop of *MpmiR160*, and *amiR-MpE(z)*^{SkMIR166} retains that of *SkmiR166*. All mismatches between the endogenous miR and miR* sequences were retained in the amiR design. miR and amiR sequences are shown in color, while miR* sequences are shown in bold and lower case. (B) Target sites of the amiRs used in this study; amiRs were designed to have perfect complementarity with their targets. The symbol indicates predicted cleavage sites by AGO proteins, located between bases 10 and 11 of the miR/amiR-binding sites. (C) amiR expression in cis using the constitutive regulatory sequences, $_{pro}$EF1. (D) amiR expression in trans, where the chimeric XVE transcription factor is driven by $_{pro}$EF1. All T-DNAs carry either *hptII*, conferring hygromycin resistance, or *nptII*, conferring resistance to G418, genes that allow selection of transgenic lines, driven by the *Cauliflower mosaic virus* 35S promoter ($_{pro}$35S). (E) Control T-DNA used in this study: left and right borders flank a dual $_{pro}$35S driving either *hptII* or *nptII*.

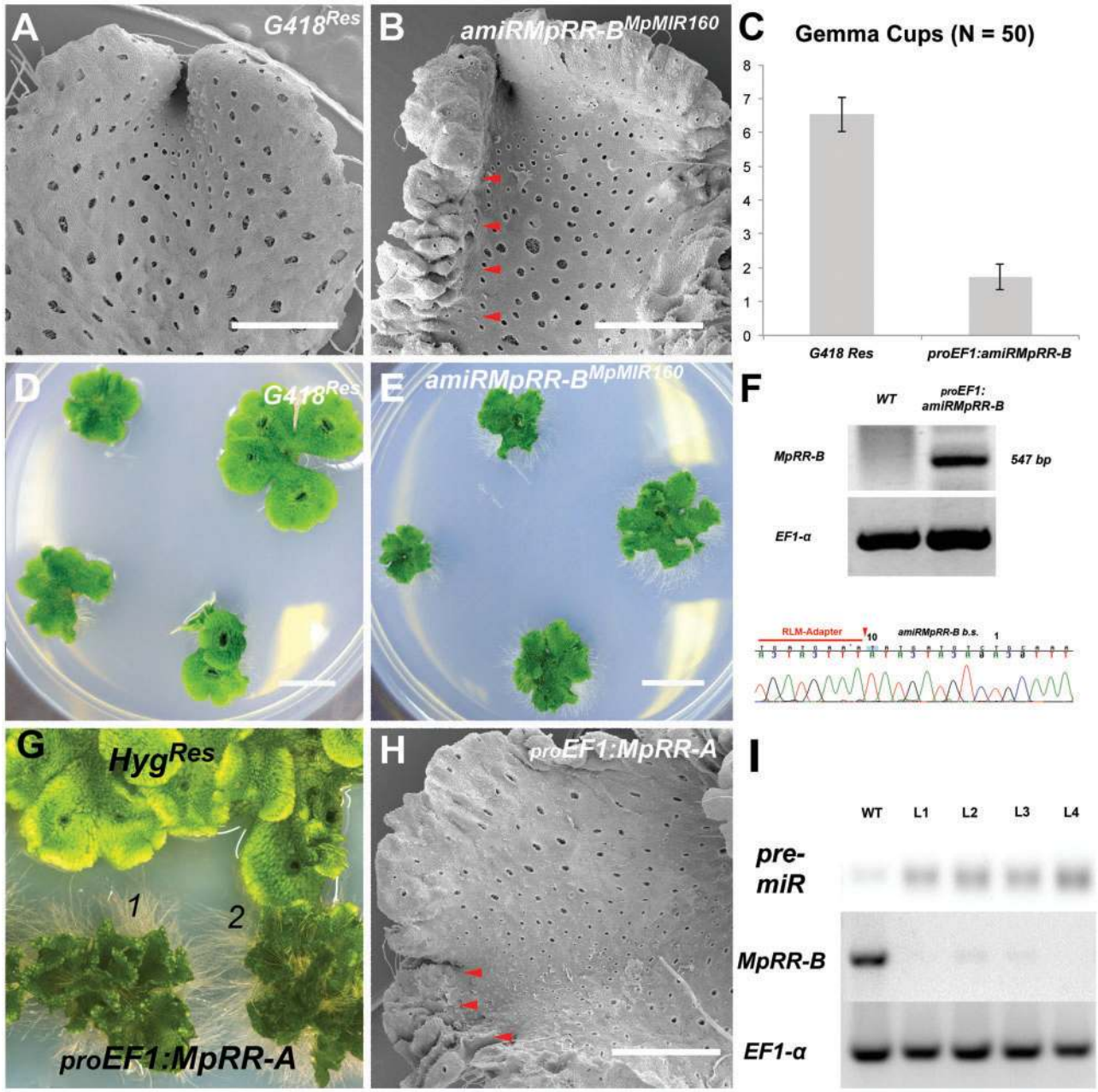


Fig. 2 *MpRR-B* knockdowns result in morphological defects in *Marchantia*. (A) Scanning electron microscopy of a wild-type thallus grown for 28 d showing air pores, smooth thallus margins and a single apical notch. (B) Representative $_{pro}$*EF1:amiR-MpRR-B*^{MpMIR160} primary transformant grown for 28 d showing convoluted thallus margins (arrowheads). (C) $_{pro}$*EF1:amiR-MpRR-B*^{MpMIR160} lines produce fewer gemmae cups as compared with the controls. Primary transformants (n = 50) were assayed after 25 d of growth in B5/G418 media. (D) Thirty-day-old primary transformants expressing G418^{Res} transgene grown on mock media. (E) Four independent 30-day-old $_{pro}$*EF1:amiR-MpRR-B*^{MpMIR160} primary transformants, simultaneously transformed with controls in (D), are smaller in size, and have profuse rhizoids and no gemmae cups. (F) RLM 5′-RACE detection of *amiR-MpRR-B*^{MpMIR160} directed cleavage of *MpRR-B*. Direct sequencing of RLM 5′-RACE fragments demonstrates that cleavage occurs between bases 10 and 11 of the *amiR-MpRR-B*^{MpMIR160}-binding site. (G) Two independent *proEF1:MpARR-A* lines displaying similar phenotypes to $_{pro}$*EF1:amiR-MpRR-B*^{MpMIR160}. (H) Scanning electron microscopy of a representative *proEF1:MpRR-A* line showing ectopic outgrowths in the margins (arrowheads). (I) Semi-quantitative RT–PCR illustrating levels of *pre/pri-amiR* and target levels (*MpRR-B*) in control and the lines observed in **Supplementary Fig. 2**D. Note that the Hyg^{Res} control has a low level of *pre/pri-miR* expression probably as a background for the endogenous *MpmiR160*. Scale bar = 1 mm. Scale bars in A, B and H = 1 mm; D, E and G = 1 cm.

Song et al. 2004). Consistent with miR-mediated knockdown of *MpRR-B*, expression of *amiR-MpRR-B*^{MpMIR160} revealed a diminished expression of its target gene, *MpRR-B*, compared with the controls, although traces of *MpRR-B* expression were detectable in plants that were able to form gemmae cups (L3) and did not exhibit thallus serrations characteristic of *amiR-MpRR-B*^{MpMIR160} overexpression (**Fig. 2I**; **Supplementary Fig. S2**D).

Table 1 Serrations in primary transformants after 38 days of growth ($n = 96$)

Phenotype	$G418^{Res}$	$_{pro}$*EF1:amiRMpRR-B*
No serrations	96	26
Mild serrations	0	7
Clear serrations	0	63

Table 2 Auxin resistance in primary transformants after 2 weeks growth on media supplemented with exogenous auxin

	Hyg^{Res}	$_{pro}$*EF1:amiRMpARF1*
Total transformants	771	299
Auxin resistant	2	54
% Auxin resistant	0.259403372	18.06020067

In *A. thaliana*, type A RESPONSE REGULATORs (RR-As) have been demonstrated to repress the cytokinin transcriptional response by competing with RR-Bs for phosphorylation by ARABIDOPSIS HISTIDINE-CONTAINING PHOSPHOTRANSFER PROTEINS (AHPs) (To et al. 2004, Kieber and Schaller, 2014). To corroborate further that $_{pro}$*EF1:amiR-MpRR-B*^{MpMIR160} lines exhibit a disruption of *MpRR-B* transcriptional activity, we constitutively overexpressed the single *M. polymorpha* RR-A ortholog (*MpRR-A*). The phenotypes of $_{pro}$*EF1:amiR-MpRR-B*^{MpMIR160} and $_{pro}$*EF1:MpRR-A* lines are similar in multiple aspects of their morphologies. First, both genotypes have ectopic serrations (or outgrowths) on thallus margins (**Fig. 2G–H**). Secondly, both genotypes produce almost no gemmae cups or aberrant gemmae cup primordia that fail to develop to full maturity (**Supplementary Fig. S2**E). Thirdly, the thallus size is diminished, exposing ventral rhizoids and giving the plants an appearance of producing ectopic rhizoids. Thus, loss-of-function *MpRR-B* alleles and gain-of-function *MpRR-A* alleles result in similar morphological abnormalities, and $_{pro}$*EF1:amiR-MpRR-B*^{MpMIR160} lines exhibit a reduced sensitivity to exogenous cytokinin, suggesting that miR-mediated knockdown of *MpRR-B* was effective.

Knockdown $_{pro}$*EF1:amiRMpARF1* lines are auxin resistant

The *M. polymorpha* class A *AUXIN RESPONSE FACTOR1* (*MpARF1*) appears to be the primary transcriptional activator of the largely conserved auxin transcriptional response pathway in *Marchantia* (Flores-Sandoval et al. 2015, Kato et al. 2015). Therefore, loss-of-function alleles of this gene result in plants unable to respond to exogenous auxin. Such a phenotype has previously been observed in *MpARF1* deletion alleles obtained by CRISPR-CAS technology (Sugano et al. 2014). Wild-type plants grown in the presence of exogenous 10 μM 2,4-D, an auxin analog, produce ectopic rhizoids, with rhizoids emanating from both dorsal and ventral surfaces (Tarén 1958, Maravolo and Voth 1966, Ishizaki et al. 2012). We plated a population of spores transformed with $_{pro}$*EF1:amiR-MpARF1*^{MpMIR160} on plates supplemented with 10 μM 2,4-D and scored plants for the production of ectopic rhizoids to facilitate rapid identification of a mutant phenotype (**Supplementary Fig. S3**A, B). In a $_{pro}$*EF1:amiR-MpARF1*^{MpMIR160} T_1 population, 54 out of 299 lines (19%) failed to produce ectopic rhizoids, as compared with only about two out of 771 lines (0.25%) of a spore population transformed with a control T-DNA expressing only a hygromycin resistance gene (**Table 2**; **Supplementary Fig. 3**A, B). Using RLM 5′-RACE and direct sequencing of whole populations of amplicons, we detected the predicted cleavage products of *MpARF1* in auxin-resistant primary transformants (**Fig. 3A**). Independent $_{pro}$*EF1:amiR-MpARF1*^{MpMIR160} lines exhibit decreases of *MpARF1* levels as assayed by semi-quantitative RT–PCR (**Fig. 3B**). Primary transformants were plated alongside Hyg^{Res} controls to screen for phenotypic variability (**Table 3**; **Supplementary Fig. S3**C–F). Despite being auxin resistant, the typical $_{pro}$*EF1:amiR-MpARF1*^{MpMIR160} line only showed a slight decrease in thallus size and gemmae cup production compared with controls (**Supplementary Fig. S3C, D**; **Table 2**). Gemmalings of an auxin-resistant $_{pro}$*EF1:amiR-MpARF1*^{MpMIR160} line were examined for sensitivity to auxin and were also found to be resistant to 10 μM 2,4-D (**Fig. 3C**). Since each gemma is derived from a single cell of the primary transformant, each gemma represents a clone of the parent plant; thus, amiR activity was stable across an asexual generation. After 45 d of growth, primary transformants also exhibited a loss of gemmae dormancy within the gemmae cups, another phenotype associated with auxin response (LaRue and Narayanaswami 1957, Maravolo and Voth 1966, Eklund et al. 2015; **Fig. 3D**). Thus, $_{pro}$*EF1:amiR-MpARF1*^{MpMIR160} lines showed phenotypes consistent with physiological responses to auxin but lacked the severe morphological defects associated with auxin depletion or null or dominant mutations that completely inhibit auxin response (Flores-Sandoval et al. 2015, Kato et al. 2015).

Inducible amiR activity allows the study of putative lethal phenotypes.

In contrast to experiments with $_{pro}$*EF1:amiR-MpRR-B*^{MpMIR160} and $_{pro}$*EF1:amiR-MpARF1*^{MpMIR160}, transformation of sporelings with $_{pro}$*EF1:amiR-MpE(z)*^{MpMIR160} resulted in few surviving lines compared with sporelings transformed with a T-DNA expressing just Hyg^{R} after 14 d under selective media (**Supplementary Fig. S4**). Based on this result, we surmised that knockdown of *MpE(z)* results in lethality at an early stage of sporeling development. A small number of viable plants were initially recovered, but these plants had a callus-like appearance and did not survive to produce a thallus. Likewise, transformations using a constitutively expressed amiR designed in the SkMIR166 background, $_{pro}$*EF1:amiR-MpE(z)*^{SkMIR166}, exhibited a similar spectrum of lethality. To circumvent the lethality caused by constitutive expression, we designed *amiR-MpE(z)*^{SkMIR166} to be inducible by placing it under the control of the XVE system. The chimeric XVE transcription factor is composed of the lexA DNA-binding domain derived from *Escherichia coli*, the estrogen-binding domain of the estrogen receptor from *Homo sapiens*, and a transcription activation domain, VP16

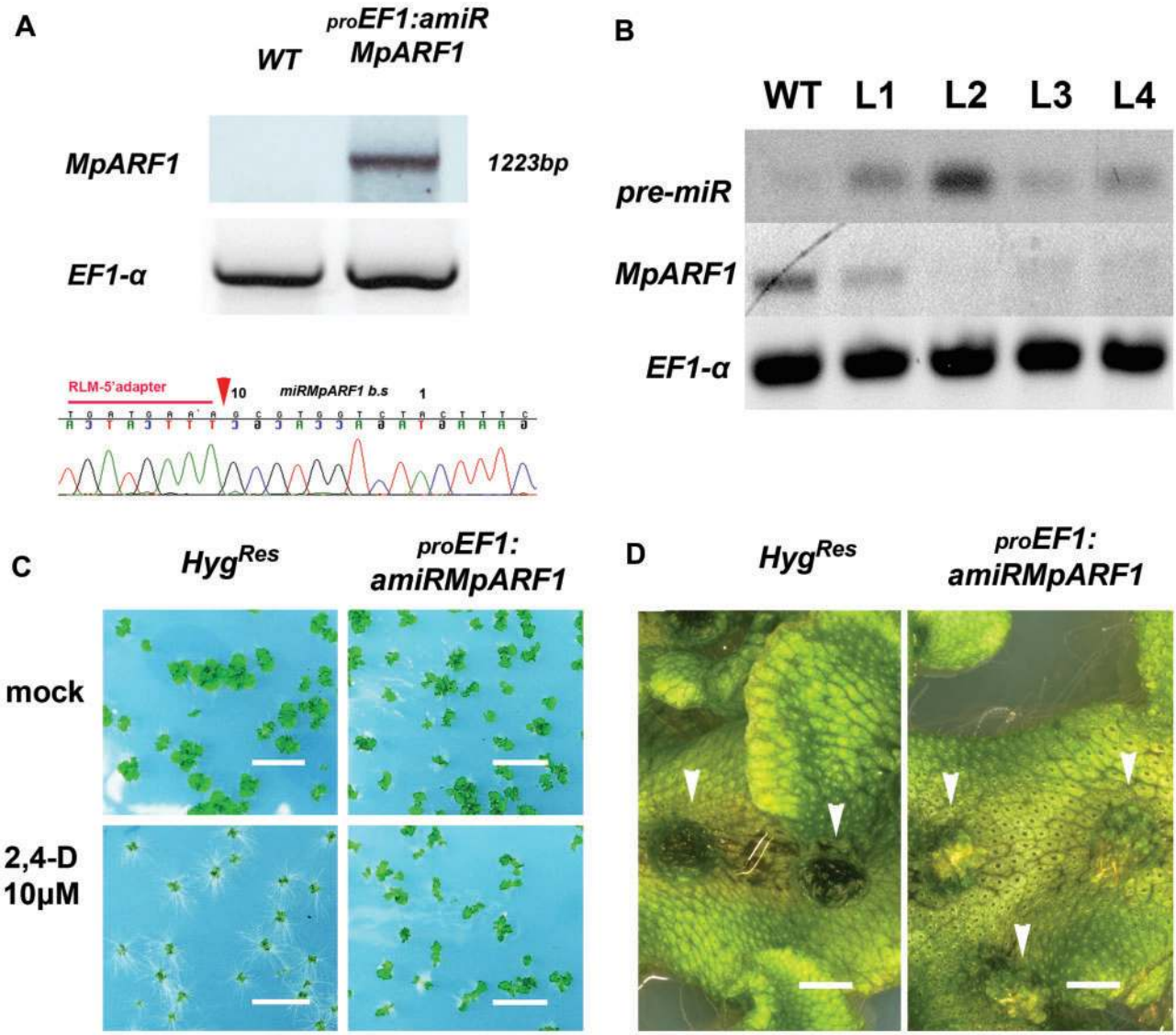


Fig. 3 *MpARF1* knockdowns disrupt physiological auxin responses in *Marchantia*. (A) RLM 5′-RACE experiments showing the cleavage product of *MpARF1* mRNA transcripts as a result of *amiR-MpARF1*^{MpMIR160} activity. Direct sequencing of the 1,223 bp fragment demonstrates that cleavage occurs between bases 10 and 11 of the amiR-binding site. *EF1-α* provides a quantitative control. (B) Semi-quantitative RT–PCR illustrates *pre/pri-amiR* and target levels (*MpARF1*) in four independent $_{pro}$*EF1:amiR-MpARF1*^{MpMIR160} primary transformants (C) Wild-type gemmalings develop ectopic dorsal rhizoids if grown in the presence of 10 µM 2,4-D for 9 d. The first gemmaling generation (G_1) of an auxin-resistant $_{pro}$*EF1:amiR-MpARF1*^{MpMIR160} primary transformant does not produce ectopic rhizoids if grown in the presence of 10 µM 2,4-D. Both gemmaling populations were grown for 9 d on exogenous auxin from day 0 of development. (D) Left panel: 45-day-old Hyg^{Res} primary transformants have dormant gemmalings resting within cups (arrowheads). Right panel: 45-day-old $_{pro}$*EF1:amiR-MpARF1*^{MpMIR160} primary transformants have non-dormant gemmalings already growing within cups (arrowheads). Scale bars in C = 1 cm and D = 1 mm.

(Zuo et al. 2000). In our system, XVE is driven by $_{pro}$EF1 and can transactivate genes with lexA regulatory binding sites [$_{pro}$*EF1:XVE > > amiR-MpE(z)*^{SkMIR166}], where both transgenes are included within a single T-DNA. We transformed spores and found most $_{pro}$*EF1:XVE > > amiR-MpE(z)*^{SkMIR166} plants to exhibit a wild-type phenotype, but with some transformants growing more slowly and showing phenotypic aberrations.

We selected phenotypically wild-type $_{pro}$*EF1:XVE > > amiR-MpE(z)*^{SkMIR166} primary transformants (T_1), and subcultured two successive asexual generations of isogenic gemmae (G_1 and G_2) from these T_1 plants. Since gemmae develop from a single cell, the gemmalings cannot be chimeric. We observed that uninduced lines had a wild-type morphology in the absence of estrogen, but they experienced arrested development and eventual lethality in the presence of 5 µM β-estradiol (**Fig. 4A**). Cleaved *MpE(z)* transcripts could not be detected in uninduced G_2 gemmalings, but were detectable after 12 h of β-estradiol induction (**Fig. 4B**). Consistent with the proposed lethal phenotype at the sporeling stages, prolonged growth of $_{pro}$*EF1:XVE > > amiR-MpE(z)*^{SkMIR166} plants in the presence of exogenous 5 µM β-estradiol also resulted in lethality.

Table 3 Phenotypic effects of $_{pro}$*EF1:amiRMpARF1* in 30-day-old primary transformants ($n = 33$)

Measurement	WT Hyg^{Res} (control)	SD	SE	*EF1:amiRMpARF1*	SD	SE	Two-tailed *t*-test
Notch average	16.82	3.22	0.56	17.12	2.73	0.47	0.682840281
Cup average	17.94	5.25	0.91	14.71	3.35	0.58	0.0041358
Cup/notch ratio	1.11	0.37	0.06	0.88	0.24	0.04	0.005669443
Area average (cm^2)	10.63	1.94	0.34	8.14	1.07	0.18	**4.21519E-08**
Area/notch ratio	0.66	0.17	0.03	0.49	0.11	0.02	**1.9847E-05**
Cups cm^{-2}	1.67	0.34	0.06	1.81	0.34	0.06	0.107134907
Notches cm^{-2}	1.64	0.49	0.09	2.14	0.46	0.08	**7.06655E-05**

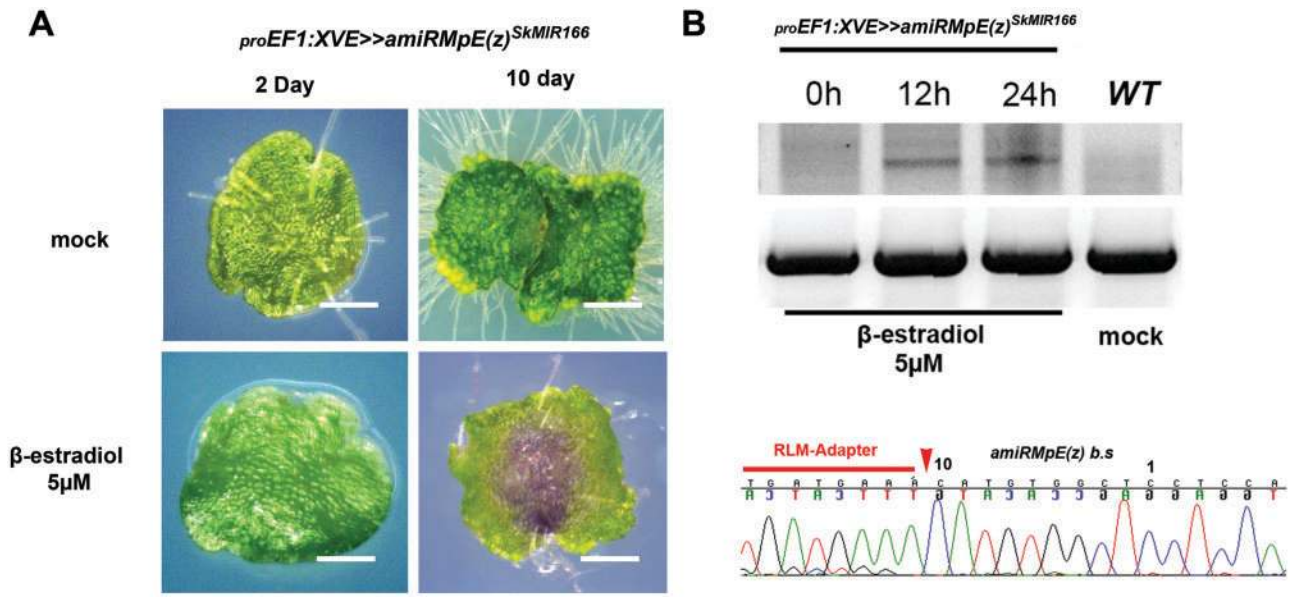


Fig. 4 Inducible expression of amiRs in *Marchantia*. (A) $_{pro}$*EF1:XVE > > amiR-MpE(z)*^{SkMIR166} gemmalings after 2 and 10 d of growth either in a mock treatment or in the presence of 5 μM β-estradiol. Necrosis is evident after 10 d of growth in the presence of the inducer. (B) RLM 5′-RACE experiments showing the cleavage product of *MpE(z)* mRNA transcripts as a result of *amiR-MpE(z)*^{SkMIR166} activity in mock- and β-estradiol-treated plants prior to and 12 and 24 h after transfer to plates containing 5 μM β-estradiol. Direct sequencing of RLM 5′-RACE fragments demonstrates that cleavage occurs between bases 10 and 11 of the *amiR-MpE(z)*^{SkMIR166}-binding site. Scale bars = 0.5 mm.

Discussion

Possible differences in miR processing between *M. polymorpha* and other land plants

Our results demonstrate the design and use of amiRs to study gene function in *M. polymorpha*. Both the endogenous *M. polymorpha MpMIR160* and the *S. kraussiana SkMIR166* precursors were successfully modified to design amiRs that were active in *M. polymorpha*. However, constitutive expression of an *A. thaliana MIR160a* precursor that has been shown to be active in *A. thaliana* was not sufficient to recapitulate the phenotype observed with constitutive expression of *MpMIR160* (**Supplementary Fig. S1**). In contrast, the *A. thaliana AtMIR319* precursor has been demonstrated to be functional in the moss *Physcomitrella patens* (Khraiwesh et al. 2008). While the machinery necessary to process miRs and down-regulate miR targets was present in the common ancestor of all land plants (Floyd and Bowman 2004, Bowman et al. 2007), there might be differences in miR processing or maturation between liverworts and other major embryophyte lineages. Directed mutation experiments changing hairpin secondary structure could pinpoint if there are taxon-specific elements necessary for miR processing in the major lineages of land plants as has been explored in other animals. For example, *Caenorhabditis elegans* pri-miRs are not efficiently processed and recognized by human cells unless motifs outside the basic pri-miR hairpin are mutagenized to match those of the host cell. Such motifs have been specifically lost in the lineage leading to *C. elegans* as it is present in other bilaterian model systems (Auyeung et al. 2013).

Spectrum of phenotypes using amiRs in *M. polymorpha*.

A significant proportion of $_{pro}$*EF1:amiR-MpARF1*^{MpMIR160}-expressing plants were auxin resistant compared with the controls (**Table 2**). However, we were not able to identify lines with strong defects in cell differentiation and patterning such as

observed in null MpARF1 alleles generated by CRISPR-CAS genome editing (Sugano et al. 2014). Thus, in the case of *MpARF1*, it is likely that none of the amiR-generated alleles are null. In contrast, the high frequency of lethality observed in $_{pro}$*EF1:amiR-MpE(z)*^{MpMIR160} and $_{pro}$*EF1:amiR-MpE(z)*^{SkMIR166} lines suggests that, at least in the case of *MpE(z)*, strong loss-of-function, if not null alleles, can be produced. That similar phenotypes can be induced by either of the backbones indicates that both can be modified to produce amiRs that act with high efficacy. Support of efficacy of amiRs to produce conspicuous morphological defects comes from $_{pro}$*EF1:amiR-MpRR-B*^{MpMIR160} lines, where 64% of plants ($n = 98$) exhibited serrated thallus margins and lacked gemmae cup formation (**Table 1**). That the loss-of-function $_{pro}$*EF1:amiR-MpRR-B*^{MpMIR160} alleles exhibit a phenotype similar to $_{pro}$*EF1:MpRR-A* alleles suggests specificity of the activity of *amiR-MpRR-B*^{MpMIR160}.

The efficient cleavage of target products could depend on miR design, position of the T-DNA insertion, whether the target gene exhibits robust feedback regulation, and the context and activity of promoters that are used to drive a particular amiR relative to the endogenous sequences regulating the targets. However, even in the cases where amiR activity creates weak loss-of-function phenotypes, amiRs could be useful to anticipate or predict knockout phenotypes created by gene targeting (Ishizaki et al. 2013).

The XVE-inducible system: challenges ahead

Since constitutive expression of *amiR-MpE(z)*^{SkMIR166} and *amiR-MpE(z)*^{MpMIR160} resulted in sporeling lethality, we used an approach to produce a conditional loss-of-function allele using an estrogen-inducible system (Zuo et al. 2000, Brand et al. 2006). We recovered viable transformants with the $_{pro}$*EF1:XVE > > amiR-MpE(z)*^{SkMIR166} construct under uninduced conditions, and these plants, when transferred to estrogen-containing media, ceased to grow and eventually perished. Our adapted version of the XVE-inducible system in *Marchantia* facilitated the detection of *amiR-MpE(z)*^{SkMIR166}-directed cleavage products of *MpE(z)*, demonstrating the activity of *amiR-MpE(z)*^{SkMIR166}. While we have demonstrated the inducible nature of gene expression with the XVE system in *M. polymorpha*, long-term maintenance of $_{pro}$*EF1:XVE > > amiR-MpE(z)*^{SkMIR166} lines suggests that most lines are 'leaky' with a low level of transgene expression in the absence of exogenous estrogen. Thus, multiple lines must be screened before selecting an inducible line with negligible leaky expression. Alternative approaches to produce conditional alleles, such as the use of heat shock or ethanol-inducible promoters, should be tested, and, in the case of nuclear proteins dexamethasone-inducible glucocortocoid receptor fusions are an option that has been successfully applied in *M. polymorpha* (Kato et al. 2015).

Potential and challenges of amiRs in *Marchantia* for reverse genetics studies.

One potential advantage of using amiRs to investigate gene function is the possibility to drive amiR expression with tissue- or cell type-specific promoters, thereby limiting the knockdown effects spatially and temporally. In angiosperm species, specific phenotypes are produced by driving expression of amiRs in spatially and temporally restricted patterns, despite the ability of some miRs to move from cell to cell, albeit in a quantitatively and qualitatively limited manner (Tretter et al. 2008, Carlsbecker et al. 2010, Felippes et al. 2011). Thus, it is possible to envisage identification of several regulatory sequences that could drive amiR expression in specific tissues, allowing detailed study of gene function in *Marchantia*. However, an understanding of the extent of miR movement in *M. polymorpha* is required before using amiRs as a tool to study patterning at the single-cell level. Furthermore, amiRs could be engineered to target multiple genes, as has been done in angiosperm model systems (Alvarez et al. 2006, Schwab et al. 2006). Overall, given the appropriate context, amiRs are capable of generating strong physiological and morphological defects in *M. polymorpha*, adding a new tool to understand gene function in this emerging model system.

Materials and Methods

Design of amiRs

We mimicked the strategy applied to amiR design in angiosperm systems (Alvarez et al. 2006, Schwab et al. 2006, Ossowski et al. 2008) where the *miR* sequences in the selected pre-miR backbone are substituted for a 21 nucleotide sequence complementary to the target gene of interest (**Supplementary Fig. S5**). The *miR* sequence (5′ to 3′) was designed to start with a U and to be GC rich from bases 17 to 19 (**Fig. 1B**). The miR* sequence was designed to have mismatches with the miR sequence in the same positions occurring in the original *MIR* precursor (**Supplementary Fig. S5**). A BLAST search of 25–30 of the putative target sequences facilitated selection of one with the fewest potential off-targets. Selection of specificity took into account that positions 2–12 of the miR sequence are critical for recognition between miR/miR-binding sites (Schwab et al. 2005). All designed amiRs were folded in silico using m-fold prior to synthesis to corroborate appropriate design (Zuker 2003). The sequences for the miR- and amiR-binding sites in this study are given in **Supplementary Fig. S6**.

Vector construction

Artificial miRs. amiRs were synthesized by Genscript® and designed to have *Kpn*I/*Hin*dIII sites to subclone from puC57 into the shuttle plasmid $_{pro}$EF1:BJ36. *Not*I/*Sca*I restriction reactions allow gel extraction of a band with a $_{pro}$*EF1:amiR:ocs* fragment and subcloning into pHART (Hyg^{Res}) and/or pKART (G418^{Res}) using its unique *Not*I site and blue/white selection.

XVE-inducible system. This vector was based on pLB12 (Brand et al. 2006). XVE was cloned into pCRII using primers XVE *Kpn*I-F (5′-ATGGTACCATGAAA GCGTTAACGGCCAGGCAAC-3′) and XVE-*Cla*I-R (5′-ATATCGATCTTGTTTG GGATGTTTTACTCCTC-3′). This was later subcloned into $_{pro}$EF1:BJ36 with *Kpn*I and selected for correct orientation using Primers EF1proF2 (5′-ACTGG AGCTTTTACGTGGTGTCGATT-3′) and XVE-*Cla*I-R. $_{pro}$EF1:XVE was amplified using EF1:XVE.NdeI-F (5′-ATCATATGCAAATGAGTCACACACATTGTTG-3′) and EF1:XVE-NdeI.R (5′-ATCATATGCTGCTGAGCCTCGACATGTTGT-3′), and subcloned into lexA-BJ36 using *Nde*I. Correct orientation was checked with *Pvu*I. A partial *Not*I digest in $_{pro}$*EF1:XVE;lexA:BJ36* (1/10 *Not*I dilution for 30 min) created a 7.425 kb fragment that did not disrupt the Gateway® recombination site downstream of the lexA operator. Such a fragment was ligated into the HART binary vector using *Not*I and blue/white selection. The new plasmid was called MpXVEindie. *amiR-MpE(z)*^{SkMIR166} was cloned into MpXVEindie with a Gateway® LR reaction.

Plant transformation and growth conditions

Constructs were transformed into *Agrobacterium* strain GV3001 and protocols were followed as described in Ishizaki et al. (2008). Plants were subjected to two rounds of selection on 1/2B5 plates containing hygromycin (10 μg ml^{-1}) and

timentin (200 μg ml^{-1}) plates and with 10 μM 2,4-D in the case of *proEF1:amiR-MpARF1* experiments. Gemma used for $_{pro}$*EF1:XVE* > > *amiR-MpE(z)*^{SkMIR166} induction were transferred to plates containing 5 μM 17-β-estradiol made from 20 mM stock in 70% ethanol (plates were kept at −20°C in the dark to prevent light dependent degradation).

Cleavage detection of targets and transcript monitoring

A QIAGEN RNA-easy kit® was used for RNA extractions from thalli tissue. Total RNA was ligated with an RLM First Choice® 5′RACE adaptor (5′-GCUGAUGGC GAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA-3′)™ using T4 RNA Ligase (NEB), and precipitated using phenol : chloroform. The resulting eluate was used for cDNA synthesis using a GAGA primer and Bioscript Reverse Transcriptase®. *MpARF1* fragments were detected in a single round of PCR using primers 5′RACE Outer (5′-GCTGATGGCGATGAATGAACACTG-3′) and MpARF1-RLM-R3 (5′-CATGACCCGATAAAAATCTGTTGGTGTACC-3′). *MpRR-B* fragments were detected in two rounds of PCR using primers 5′RACE Outer and MpRR-B-RLM-R2 (5′- TTGGTGAGGGAAGACTTGGTTCA ATAAGG-3′) and 5′RACE inner (5′-CGCGGATCCGAACACTGCGTTTGCTGG CTTTGATG-3′) and MpRR-B-RLM-R1 (5′- AATCTGCATCGTACCACCAAAGT TCGTTCC-3′). *MpE(z)* fragments were detected in one round of PCR using 5′RACE Outer and *MpE(z)* 5′Rout (5′-GCTTCGGTGATGGAAGCTTTGAGTA ACTTC-3′). amiRs were monitored with semi-quantitative RT–PCR from thallus cDNA using primers Inf-amiRMp160-F2 (5′-GGTACCGCACCTCCTCTCTCC GAC-3′) and amiRMp160-F2 (5′-AAGCTTTAAGTAAATCTATCAAACATCAG-3′). MpRR-B transcript levels were monitored with semi-quantitative RT-PCR using MpRR-B-F (5′-GGTAC**C**ATGATGAAGTCATTTGATACAGCCACCTC-3′) and MpRR-B-R (5′-GGATCCTCACTGACCCTGACCAGGCCTATAATCGTC-3′). MpARF1 transcript levels were monitored using MpARF1ATGF-KpnI (5′GGTACC**ATG**TATTCTTGTTCGCCGATGAGGCTG3′) and MpARF1-RLM-R3. The EF1 control was amplified using primers MpEF1-CDS-F (5′-ATGCATCTCGACGG ACTTGACCTC-3′) and MpEF1-CDS-R (5′-TTCAAGTACGCCTGGGTGCTCGAC-3′).

Microscopy

Plants were observed in a Lumar.V12 dissecting microscope (Zeiss) and photographed with an AxioCam HRc (Zeiss). Fixation for scanning electron microscopy was performed in FAA (50% ethanol, 5% acetic acid, 10% formaldehyde) overnight at 4°C. Samples were dehydrated in ethanol before critical point drying in a CPD 030 (Baltec). Samples were sputter coated with gold using a SCD 005 (Baltec). Scanning electron microscopy was performed with an S-570 microscope (Hitachi) at 10 kV and photos were digitalized using SPECTRUM® Software (Dindima).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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