**MpWIP regulates air pore complex development in the liverwort *Marchantia polymorpha***

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**Summary statement**

The MpWIP gene controls the development of the air pore complex – a multicellular structure that increases CO₂ uptake – in the early-diverging land plant *Marchantia polymorpha*.

**Keywords**

*Marchantia polymorpha*, air pore complex, WIP protein
ABSTRACT

The colonisation of the land by plants was accompanied by the evolution of complex tissues and multicellular structures comprising different cell types as morphological adaptations to the terrestrial environment. Here we show that the single WIP protein in the early-diverging land plant *Marchantia polymorpha* L. is required for the development of the multicellular gas exchange structure, the air pore complex. This 16-cell barrel-shaped structure surrounds an opening between epidermal cells that facilitates the exchange of gasses between the chamber containing the photosynthetic cells inside the plant and the air outside. MpWIP is expressed in cells of the developing air pore complex and the morphogenesis of the complex is defective in plants with reduced MpWIP function. The role of WIP proteins in the control of different multicellular structures in *M. polymorpha* and the flowering plant *Arabidopsis thaliana* suggests that these proteins controlled the development of multicellular structures in the common ancestor of land plants. We hypothesise that WIP genes were subsequently co-opted in the control of morphogenesis of novel multicellular structures that evolved during the diversification of land plants.
INTRODUCTION

Morphological diversity increased dramatically after plants colonised the land some time before 460 million years ago (Kenrick and Crane, 1997). The evolution of unicellular and multicellular structures with specialised functions in the outermost cell layer – the epidermis – provided plants with the means to increase the surface area over which CO₂ uptake from the atmosphere occurred, and to extract water and inorganic nutrients from the early soil. Some specialized epidermal structures are present in all extant lineages of land plants. For example, tip-growing rhizoids and root hairs emerge from the epidermis to provide anchorage and take up water and nutrients from the soil (Jones and Dolan 2012). The phylogenetic distribution of others is more restricted; stomata, valves in the epidermis consisting of two specialized guard cells that open and close to regulate gas exchange, develop in all land plant lineages except the early-diverging Marchantiophyta (liverworts). In one group of liverworts, the Marchantiidae, the evolution of complex tissues has been accompanied by an independent evolution of a multicellular epidermal structure that facilitates gas exchange, the air pore complex (Crandall-Stotler et al., 2009). We report here that the zinc finger protein MpWIP is necessary for the morphogenesis of the air pore complex in the epidermis of Marchantia polymorpha.

RESULTS AND DISCUSSION

A gain-of-function mutation in MpWIP causes defective development of the dorsal epidermis

To identify genetic mechanisms controlling the development of specialised morphological structures that operated in the earliest land plants we screened for mutants with defects in the development of epidermal structures in the liverwort Marchantia polymorpha, a member of one of the earliest-diverging groups of land plants. Multicellular air pore complexes, gemma cups and gemmae develop on the dorsal epidermis of M. polymorpha (Fig. 1 A,C) while unicellular rhizoids and multicellular membranous outgrowths (scales) develop on the ventral epidermis (Fig. 1 B,D). In a screen of T-DNA insertion mutants (Honkanen et al., 2016) we isolated a mutant, vj7, that develops rhizoids from the epidermal cells of the mature dorsal epidermis (3.76 rhizoids/mm², n=5); rhizoids do not develop on the dorsal epidermis.
of the wild type (Fig. 1 E,F). The dorsal rhizoid phenotype of mutant \textit{vj7} results from a single mutation that is tightly linked to a T-DNA insertion 764 bp upstream of the transcriptional start site of a gene (Supplemental Data 1) encoding a member of the WIP zinc finger protein family, MpWIP (GenBank: KX645870) (Fig. 1 G, Figs S1,S2).

We hypothesised that the T-DNA insertion in \textit{vj7} would impact the transcription of the Mp\textit{WIP} gene 3' from the T-DNA right border. To quantify the effects of this insertion on Mp\textit{WIP} expression, we measured the steady-state levels of Mp\textit{WIP} transcript in the wild type and mutant \textit{vj7}. Mp\textit{WIP} transcript level was almost four times higher in \textit{vj7} than in the wild type Tak-2 (Fig. 1 H), consistent with the hypothesis that \textit{vj7} is a gain-of-function Mp\textit{wip} mutant. To independently verify that Mp\textit{WIP} gain-of-function induces the development of rhizoids on the dorsal surface of \textit{M. polymorpha}, we expressed Mp\textit{WIP} under the control of the constitutively-active Os\textit{ACTIN} promoter (\textit{pro} Os\textit{ACT}:Mp\textit{WIP}) (Breuninger et al., 2016), and isolated a line in which the level of Mp\textit{WIP} transcript is twice that seen in the wild type (Fig. 1 H). Plants of this line developed ectopic rhizoids on the dorsal surface, as observed in \textit{vj7} but not the wild type Tak-2 (Fig. 1 I). This is consistent with the hypothesis that a gain of Mp\textit{WIP} function causes the development of ectopic rhizoids in mutant \textit{vj7}. We conclude that \textit{vj7} is a gain-of-function mutant of Mp\textit{WIP} and designated it Mp\textit{wip-1GOF}.

The Mp\textit{WIP} promoter is active in developing air pores

To investigate where the Mp\textit{WIP} promoter is active in the wild type we expressed 3\textit{xYFP-NLS} under the control of a 4.7 kb fragment of genomic DNA upstream of the CDS of \textit{MpWIP} (\textit{pro}Mp\textit{WIP}:\textit{YFP-NLS}). In plants transformed with \textit{pro}Mp\textit{WIP}:\textit{YFP-NLS} fluorescent protein was detected in cells in the apical region of both the ventral and dorsal sides of the thallus (Fig. 2 A). The activity of the promoter in the ventral apical region, where rhizoids initiate, is consistent with a possible role for Mp\textit{WIP} in promoting rhizoid development. On the dorsal side of the thallus the Mp\textit{WIP} promoter was most active in cells of developing air pore complexes (Fig. 2 A), with lower activity in the surrounding epidermal cells. Air pores initiate as schizogenous openings that form in the epidermis at points where four cells meet (Apostolakos and Galatis, 1985a). The four cells surrounding each opening divide periclinally and
differentiate to form the multiple tiers of the barrel-shaped air pore (Fig. 2 B) (Apostolakos and Galatis, 1985a). Air chambers form below the air pores and consist of schizogenous intercellular cavities in which filaments of photosynthetic cells develop (Apostolakos and Galatis, 1985b; Ishizaki et al., 2013; Mirbel, 1835). Low levels of \( \text{pro}\text{MpWIP} \) activity were detected in all cells near the apex before air pore differentiation is visible, and this activity increased in the dividing cells of the developing air pore complex. The strong promoter activity in cells of the air pore complexes compared to surrounding cells is first apparent at the four-cell stage, when the cells surrounding the schizogenous opening first enlarge relative to the surrounding epidermal cells (Fig. 2 A,B). Strong expression continues during the periclinal divisions that generate the tiered 16-cell air pore complex (Fig. 2 A,B). The activity of the MpWIP promoter during the formation of air pore complexes suggested that MpWIP could be involved in their development.

**MpWIP is required for air pore development**

To determine if MpWIP is required for rhizoid or air pore complex development we generated plants with decreased MpWIP function. We expressed two different artificial microRNAs based on Mp\( \text{miR160} \) (Flores-Sandoval et al., 2016) that target either the 3’ UTR (\( \text{amiR-MpWIP-3’ UTR}_{\text{MpmiR160}} \)) or CDS (\( \text{amiR-MpWIP-CDS}_{\text{MpmiR160}} \)) of MpWIP under the control of \( \text{proOsACT} \). Steady-state levels of MpWIP transcript are reduced to approximately half wild type levels in plants transformed with \( \text{proOsACT}:\text{amiR-MpWIP-3’ UTR}_{\text{MpmiR160}} \) or \( \text{proOsACT}:\text{amiR-MpWIP-CDS}_{\text{MpmiR160}} \) (Fig. 3 A,B). The formation of the air chambers is delayed or abolished in the MpWIP knockdown lines, and consequently the reticulated pattern of dark green air chambers characteristic of the wild type is absent (Fig. 3 A). Furthermore, the regular 16-cell structure of the wild-type air pore complex does not develop (Fig. 3 C). Air pore development begins with the formation of schizogenous openings at the point where four cells meet, exactly as it does in the wild type (Fig S 4 A). However, the periclinal divisions that form the tiers of the air pore complex in wild type mostly fail to occur in the knockdown lines. Instead cells divide anticlinally, forming a single tier of more than four cells surrounding the pore (Fig S 4 B). This indicates that reducing the level of MpWIP transcript disrupts air pore morphogenesis after the four cell
stage, consistent with a role of MpWIP in air pore complex and air chamber development suggested by the activity of $p_{\text{WIP}}$ MpWIP during air pore development (Fig. 2 A). We were unable to quantify rhizoid density, but rhizoid development was indistinguishable from the wild type. Together these data indicate that MpWIP activity is required for the differentiation of air pore complexes, but do not provide evidence that it is necessary for rhizoid development.
**MpWIP may act as a transcriptional repressor**

At least one WIP protein, AtNO TRANSMITTING TRACT (AtNTT), binds DNA (Marsch-Martínez et al., 2014). To determine if MpWIP promotes rhizoid identity and air pore complex development through transcriptional activation or repression, we expressed chimeric dominant repressor and activator versions of MpWIP separately in transgenic plants. To generate the dominant repressor, we fused an SRDX repressive domain (Hiratsu et al., 2003) to the C-terminus of MpWIP, and to make the dominant activator we fused a VP16 activator domain to the C-terminus (Liu and Stewart, 2016; Sadowski et al., 1988; Wilde et al., 1994). Each of these fusion proteins was expressed using the constitutive CaMV 35S promoter (pro35S:MpWIP-SRDX and pro35S:MpWIP-VP16). If MpWIP promotes rhizoid and air pore differentiation via transcriptional repression, we predicted that (i) supernumerary rhizoids would develop on plants that express MpWIP-SRDX, as observed in plants overexpressing MpWIP function (Fig. 1 E,F,H,I) and (ii) plants expressing MpWIP-VP16 would develop a defective air pore phenotype similar to that caused by a loss of MpWIP function, in proOsACT:amiR-MpWIP-3’ UTRMpR160 and proOsACT:amiR-MpWIP-CDSMpR160 lines (Fig. 3 A,B,C).

Plants transformed with pro35S:MpWIP:SRDX that expressed the transgene (Fig. 4 A) developed a dense growth of ectopic rhizoids on the dorsal surface of the thallus, while air pore development was similar to wild type (Fig. 4 C). This is similar to the phenotype of the MpwipGOF mutant and proOsACT:MpWIP line (Fig.1 E,F,I). The expression of a repressive form of MpWIP therefore results in the development of plants that are morphologically similar to plants that overexpress native MpWIP, consistent with the hypothesis that MpWIP is a transcriptional repressor. Plants that express the MpWIP-VP16 transgene (Fig. 4 C) developed phenotypic defects comparable to those in lines with reduced MpWIP function, where air chamber (Fig. 4 D, Fig. 3 A) and air pore complex development are defective (Fig. 4 E, Fig. 3 C). This suggests that expression of a form of MpWIP that promotes transcriptional activation has developmental effects similar to a loss of MpWIP function. Therefore, the phenotypes of both MpWIP:SRDX and MpWIP:VP16 lines are consistent with the
hypothesis that MpWIP promotes the morphogenesis of air pore complexes through transcriptional repression.

We conclude that MpWIP is necessary for the morphogenesis of the multicellular air pore complex in the dorsal epidermis of *M. polymorpha*; air pore morphology is defective in plants with reduced WIP activity. WIP genes are also required for the development of various multicellular structures in the angiosperm *A. thaliana*. For example, AtNTT is a WIP protein required for the development of the replum, a structure that facilitates dehiscence and seed dispersal from *A. thaliana* fruits (Marsch-Martínez et al., 2014) – cell number is reduced in the repla of Atntt mutant fruits compared to wild type. Roots do not form in Atntt Atwip4 Atwip5 triple mutants, demonstrating a requirement for these three related WIP proteins in the development of the distal stem cells of the root during embryogenesis (Crawford et al., 2015). Incomplete veins form in Atdefectively organised tributaries5 (Atdot5) mutants, indicating the requirement of the WIP protein AtDOT5 in leaf vein development (Petricka et al., 2008). The demonstration that WIP proteins control the development of different multicellular structures in both early-diverging land plants and angiosperms (the latest-derived land plants) leads us to propose that WIP proteins controlled the development of multicellular structures in the common ancestor of *M. polymorpha* and *A. thaliana*, a close relative of the earliest land plants. We hypothesise that the subsequent duplication of *WIP* genes and neofunctionalisation of WIP proteins promoted the development of novel multicellular structures that evolved as the morphologies of land plants diversified.
MATERIALS AND METHODS

See Supplemental Information for primer sequences and plasmid construction.

Plant material and growth

Tak-1 male and Tak-2 female wild type accessions (Ishizaki et al., 2008) were used in this study. Mutant *vj7* was isolated in a mutant screen of spores from a cross between Tak-1 and Tak-2 transformed with the T-DNA vector pCambia1300 (Honkanen et al., 2016). Plants were grown as previously described (Honkanen et al., 2016).

Microscopy

Images were obtained using a Leica M165FC stereomicroscope, Leica M series Plan APO 1.0x objective and Leica DFC310 FX camera. For confocal microscopy plants were stained with 15 µM propidium iodide for 15 minutes, then submerged in water. Images were acquired with a Leica SP5 confocal microscope using a Leica HCX APO 40x/0.80 W U-V-I dipping lens with sequential scans. YFP fluorescence was detected using excitation at 514 nm with an argon laser and emission was measured between 524 and 568 nm using an Acousto-Optic Tunable Filter. PI was excited at 543 nm using a helium-neon laser and emission measured between 568 and 659 nm. Images were processed using FIJI to create brightest-point 3d projections (Schindelin et al., 2012).

For scanning electron microscopy samples were fixed in dry methanol, critical point dried using a Tousimis Autosamdrí-815, mounted on aluminium stubs and coated with a gold/palladium mixture using a Quorum Technologies SC7640 sputter coater. Samples were imaged immediately with a JEOL JSM-5510 SEM.

Molecular analysis of mutant *vj7* and gene expression analysis

Genomic sequences flanking T-DNA insertions were isolated by TAIL-PCR as previously described (Proust et al., 2016). Genes near the site of the insertion linked to the mutant phenotype in line *vj7* were identified using the blastn algorithm, with 5 kb of genomic sequence 3’ and 5’ to the insertion site as the template, to query an *M. polymorpha* transcriptome (Honkanen et al., 2016). RNA extraction, cDNA synthesis and quantitative PCRs (qPCRs) were carried out as previously described.
MpACT and MpAPT were used as reference genes (Saint-Marcoux et al., 2015).

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AUTHOR CONTRIBUTIONS

Conceptualization, LD; Methodology, VASJ, LD; Investigation, VASJ; Writing – Original Draft, VASJ, LD; Writing – Review and Editing, VASJ, LD; Supervision, LD; Funding Acquisition, LD
REFERENCES


of reference genes for real-time quantitative PCR experiments in the liverwort


Figures
Figure 1  A gain-of-function mutant of MpWIP develops ectopic rhizoids on the dorsal surface. A) Air pores and gemma cups (arrowhead) are produced on the dorsal thallus surface. Scale 1 mm, apex at top. B) Scales (arrow) and rhizoids (arrowhead) are produced on the ventral thallus surface. Scale 1 mm, apex at top. C) Detail of air pore complex. Scale 20 μm. D) Detail of ventral rhizoid patch. Cells that will develop into rhizoids (yellow outlines) are separated by non-rhizoid cells. Scale 20 μm. E) Rhizoids develop on the dorsal surface of older parts of the mature thallus of vj7 but not wild type. 43 d, scale 500 μm. F) Sporelings of vj7 produce rhizoids on the oldest part of the dorsal thallus surface (arrowhead). This region of wild type sporelings lacks rhizoids. 28 d, scale 2 mm. G) The T-DNA insertion that cosegregates with the mutant phenotype in vj7 is located 5' to MpWIP. Boxes represent exons; black are CDS, grey are untranslated regions. H) MpWIP transcript levels are greater in mutant vj7 and pro OsACT: MpWIP than wild type Tak-2. 14 d gemmalings. I) Expression of MpWIP driven by the constitutive promoter proOsACT causes the development of ectopic rhizoids (arrowheads), as in mutant vj7. 10 d gemmalings, scale 1 mm.
Figure 2  The MpWIP promoter is active in the ventral apical region and in developing air pores. A) Apical region of the ventral (top row) and dorsal (bottom row) surface of the thallus of proMpWIP:3xYFP-NLS. 9 d gemmaling, scale 100 µm, apex at arrowhead. B) Schematic of the stages of air pore development. A schizogenous opening develops at the point where four epidermal cells meet (stages 1 and 2). Periclinal divisions then give rise to a stack of rings, each consisting of four cells (stages 3 and 4). Surface view (left) and cross section (right). After (Apostolakos and Galatis, 1985a).
Figure 3  Reduced MpWIP expression causes defects in air pore development. A) The dark green air chambers seen in the wild type (Tak-1, Tak-2) do not develop in plants transformed with $\text{proOsACT:amiR-MpWIP-3'}$ $\text{UTR}^{\text{MpmiR160}}$ or $\text{proOsACT:amiR-MpWIP-CDS}^{\text{MpmiR160}}$. 10 d gemmalings, scale 1 mm. B) MpWIP transcript levels are reduced in lines transformed with $\text{proOsACT:amiR-MpWIP-3'}$
UTR$_{Mp^mR160}$ or proOsACT:amiR-MpWIP-CDS$_{Mp^mR160}$. 10 d gemmalings. C) Plants with reduced MpWIP transcript levels develop air pores with defective morphology, lacking the regular 16-cell air pore complex structure that develops in the wild type (Tak-2). CSLM, PI stained, 9 d gemmalings, scale 100 μm.
A

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B

- Tak-2
- MpWIP-SRDX 1
- MpWIP-SRDX 2
- MpWIP-SRDX 3

D

- Tak-2
- MpWIP-VP16 1
- MpWIP-VP16 2
- MpWIP-VP16 3

E

- Tak-2
- MpWIP-VP16 1

Scale bars: 100 μm
Figure 4   Expression of the dominant repressor MpWIP-SRDX or the dominant activator MpWIP-VP16 causes the development of ectopic rhizoids or defective air pores, respectively. A) MpWIP:SRDX transcript is detected in lines pro35S:MpWIP:SRDX 1 and 2 but not pro35S:MpWIP-SRDX 3 or Tak-2. 12 d gemmalings. B) MpWIP-VP16 transcript is detected in lines pro35S:MpWIP-VP16 1 and 2 but not pro35S:MpWIP-VP16 3 or Tak-2. 10 d gemmalings. C) The lines that express MpWIP-SRDX (pro35S:MpWIP-SRDX 1 and 2) develop ectopic rhizoids on the dorsal surface (arrowheads). 12 d gemmalings, scale 2 mm. D) Air chamber development is defective in lines that express MpWIP-VP16 (pro35S:MpWIP-VP16 1 and 2). 10 d gemmalings, scale 1mm. E) Air pore complex morphology is aberrant in lines that express MpWIP-VP16. CSLM, PI stained, 10 d gemmalings, scale 100 μm.
Supplementary Information for Jones and Dolan

MpWIP regulates air pore complex development in the liverwort *Marchantia polymorpha*

**Supplementary Data 1. Genetic analysis of mutant *vj7***

We previously reported that gain of function mutations in *MpRSL1* cause the development of rhizoids on the dorsal surface (Proust et al., 2016). To rule out the possibility that a mutation in the *MpRSL1* gene in the *vj7* background was responsible for the development of ectopic rhizoids we crossed *vj7* to two *Mprsl1*GOF mutants, *Mprsl1*GOF6 (*vj4*) and *Mprsl1*GOF7 (*vj5*). In the F1 generation resulting from the cross between *Mprsl1*GOF6 and *vj7*, 64 of 297 plants (22 %) were wild type for rhizoid development and 233 (78%) developed dorsal rhizoids. In the F1 generation of the cross between *Mprsl1*GOF7 and *vj7* 52 of 186 plants (28%) were wild type and 134 were mutant (72%). The presence of plants with a wild type phenotype among the progeny in the F1 generation indicates that the phenotypic defects in *vj7* and *Mprsl1* are due to mutations of different genes and are therefore not allelic.

To establish whether the ectopic rhizoid trait of mutant *vj7* is controlled by a single Mendelian locus we determined its inheritance. We crossed the mutant to the wild type line Tak-1 and scored the phenotypes of the next generation. Because the phenotype is expressed in the haploid stage, we expected that if the mutant phenotype was caused by a single mutation we would observe a 1:1 ratio of mutant to wild type in the F1 generation. Of 293 F1 plants scored, 131 expressed the mutant phenotype and 162 expressed the wild type phenotype (segregation ratio 1:1.24, $\chi^2$ p=0.07). Having demonstrated that the dorsal rhizoid phenotype of the *vj7* line is controlled by a single genetic locus we set out to determine if it was caused by a T-DNA insertion. The population from which mutant *vj7* was selected was generated by insertional mutagenesis using a T-DNA that confers resistance to hygromycin. If the mutant phenotype always cosegregates with hygromycin resistance, indicating genetic linkage, the insertion is likely to be responsible for the defective phenotype of the mutant. To determine if the mutation causing the development of ectopic rhizoids is linked to the T-DNA we scored hygromycin resistance in the F1 progeny. 100% of the plants that developed dorsal rhizoids were hygromycin resistant, indicating that the mutant phenotype cosegregates with hygromycin resistance. However, 101 (62%) of the wild type phenotype plants were also resistant to hygromycin, indicating that more than one insertion was segregating in the F1 population.

To identify the T-DNA insertion that cosegregates with the mutant phenotype of *vj7* we used TAIL-PCR as previously described (Proust et al., 2016) to find the genomic locations of two T-DNA insertions in *vj7*. We determined the genotype for these insertions of 106 hygromycin-resistant individuals resulting from the cross between *vj7* and the wild type Tak-1, 72 with the mutant phenotype and 34 with the wild type phenotype. One of the insertions (Fig. 1 G, main text) was found in all of the plants.
the dorsal rhizoid mutants but in none of the wild type plants, indicating that this insertion is linked to the mutant phenotype. The second insertion was found in all 34 progeny with wild type phenotype and 29 of the 72 of the mutant phenotype progeny (40%), indicating that the second T-DNA is not linked to the mutant phenotype. Taken together these data indicate that the dorsal rhizoid phenotype in the vj7 line results from a single mutation that is closely linked to the T-DNA insertion shown in Fig. 1 G (main text).
Figure S1

An alignment of the WIP domain of MpWIP and all WIP proteins from Physcomitrella patens, Selaginella moellendorffii, Oryza sativa, Aquilegia caerulea and Arabidopsis thaliana, and related non-WIP C2H2 zinc finger transcription factors from M. polymorpha and A. thaliana, AtSENSITIVE TO PROTON RHIZOTOXICITY 1 (AtSTOP1), MpSTOP1, AtJACKDAW (AtJKD) and MpINDETERMINATE(ID)-DOMAIN 2 (MpIDD2). The alignment is provided in FASTA format in Supplementary File 1.
Figure S2

Maximum-likelihood phylogeny of land plant WIP proteins. From the alignment in Figure S1 and Supplemental File 1. Nodes are marked with aLRT values. The *M. polymorpha* protein MpWIP falls into a well-supported clade containing all the WIP proteins from other species (aLRT value = 98).
Air pore density in the wild type Tak 2 and the Mpwip gain of function mutant vj7. Non air pore epidermal cells were counted in 15 mature air chambers for 7 individuals of each genotype. No significant difference in air pore density was observed (Student’s T test, p = 0.28). Error bars indicate standard error of the mean.
Early stages of air pore complex development in *pro*OsACT:amiR-MpWIP-3′ UTR*Mp.miR160*. A) As in the wild type, air pore complexes initiate as packets of four epidermal cells (outlined and shaded red). At the point where they meet a schizogenous opening forms. B) The cells surrounding the air pore opening do not divide periclinally, as they do in the wild type. Extra anticlinal divisions occur in these cells, resulting in a single tier of more than four cells. Both panels CSLM, PI stained, 10 d gemmalings, apex at top.
**Supplementary Experimental Methods**

**Plasmid construction**

See “List of oligonucleotides used in this study”, below, for sequences of primers.

**Constitutive expression of MpWIP**

For constitutive expression of MpWIP, MpWIP, including the 3’ and 5’ UTRs, was amplified from wild type genomic DNA using Phusion DNA polymerase with primers MpWIP-F and MpWIP-R and recombined into the pCR8/GW/TOPO Gateway entry vector (Invitrogen). To create the pro OsACT:MpWIP expression construct, an LR reaction was carried out between the entry vector and the plasmid proOsACT:GATEWAY:TERM:pmGW207 (Breuninger et al., 2016).

**Generation of proMpWIP:3xYFP-NLS**

To generate the proMpWIP:YFP-NLS expression vector, 4.7 kb of sequence 5’ to the start of the CDS of MpWIP was amplified in 4 overlapping segments using Phusion DNA polymerase with the primer pairs proMpWIP1-F and proMpWIP1-R; proMpWIP2-F and proMpWIP2-R; proMpWIP3-F and proMpWIP3-R; and proMpWIP4-F and proMpWIP4-R. These fragments were joined by overlap PCR using Phusion High-Fidelity DNA Polymerase (New England Biolabs) and primers proMpWIP4-F and proMpWIP1-R, and subcloned into pGEM-T.

The In-Fusion HD Cloning Kit (Clontech Laboratories) was used to introduce this promoter into the binary vector Vp57, based on the plasmid pCambia1300 with the addition of a terminator sequence 3’ to the CaMV 35S promoter that drives expression of the hpt gene. The promoter was amplified with the primers proMpWIPInFusion-F and proMpWIPInFusion-R, which add 16 bp of sequence homologous to the desired insertion site on either side, as well as a SacI site at the 3’ terminus of the promoter, and the In-Fusion reaction was performed with Vp57 linearised with Smal (New England Biolabs). The resulting plasmid was digested with SacI (New England Biolabs), dephosphorylated with Antarctic Phosphatase (New England Biolabs), and the Gateway Vector Conversion System (Thermo Fisher) was used to ligate GW Cassette C.1 in between the promoter and terminator, to generate a destination vector containing proMpWIP:GW:Term. An LR reaction was carried out between this vector and the plasmid “NLS-3xYFP in pENTRY3c” (Breuninger et al., 2016) to create an expression vector containing proMpWIP:3xYFP-NLS:Term.

**Constitutive expression of MpWIP-SRXD and MpWIP-VP16 fusion proteins**

To generate a fusion between the EAR-motif repression domain (SRDX) and the C-terminus of MpWIP, the MpWIP CDS was amplified from the pro OsACT:MpWIP expression vector with Phusion High-Fidelity DNA Polymerase and the primers MpWIP-CDS-F and MpWIP-SRXD-R. MpWIP-SRXD-R replaces the STOP codon of MpWIP with sequence encoding the SRDX domain (LDLDLELRLGFA*) (Hiratsu et
This product was recombined into the pCR8/GW/TOPO Gateway entry vector to create the MpWIP-SRDX entry vector.

To generate a fusion between the VP16 activation domain and the C-terminus of MpWIP, the MpWIP CDS was amplified from the pro OsACT:MpWIP expression vector with Phusion High-Fidelity DNA Polymerase and the primers MpWIP-CDS-F and MpWIP-VP16-R. MpWIP-VP16-R replaces the STOP codon of MpWIP with sequence encoding the VP16 domain (DALDDFDLEML*) (Seipel et al., 1994). This product was recombined into the pCR8/GW/TOPO Gateway entry vector to create the MpWIP-VP16 entry vector.

An LR reaction was carried out between each of these entry vectors and the destination vector “pro35S:GATEWAY:TERM-pCAM” (Breuninger et al., 2016).

MpWIP artificial microRNA

The MpmiR160 pre-miR backbone was used as the basis of amiR design (Flores-Sandoval et al., 2016), with the endogenous miR160 sequence replaced with 21 nt targeting the MpWIP transcript. miRs were designed using the WMD3 software (http://wmd3.weigelworld.org/) with full-length MpWIP transcript as the target. The highest-ranked amiRs targeting the 3’ UTR and CDA of MpWIP, were chosen. The amiR* was designed to have mismatches with the amiR sequence at positions 7, 13 and 18, following the recommendations of Flores-Sandoval et al., (2016). These were then used to replace the native miR160 miR and miR* sequences in the backbone. These sequences were each bracketed by attB1 and attB2 sites and synthesised by Life Technologies. These were recombined with plasmid proOsACT:GATEWAY:TERM-pMpGW207 (Breuninger et al., 2016) using LR Clonase II (Thermo Fisher) to generate the expression clones proOsACT:amiR-MpWIP-3’ UTRMpmir160 and proOsACT:amiR-MpWIP-CDSMpmir160.

Phylogenetic analysis

The genomic sequence of MpWIP was obtained from an M. polymorpha genome prepared from Tak-1 and Tak-2 accessions (Honkanen et al., 2016). This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession LVLJ00000000. The version described in this paper is version LVLJ01000000. The MpWIP transcript sequence was obtained from an M. polymorpha gametophyte transcriptome prepared from Tak-1 and Tak-2 accessions. This Transcriptome Shotgun Assembly project has been deposited at DDBJ/ENA/GenBank under the accession GEFO00000000. The version described in this paper is the first version, GEFO01000000.

To identify homologues in other land plants of the gene linked to the mutant phenotype in mutant vj7 we used the translation of the longest open reading frame to query the Arabidopsis genome using the blastn algorithm. The most similar match
was **AtNO TRANSMITTING TRACT (AtNTT)** which was 71% identical over the entire length of the protein and a member of the WIP family of zinc finger proteins.

MAFFT v. 7 (Katoh and Standley, 2013) was used to align the sequences of MpWIP and WIP proteins from Physcomitrella patens, Selaginella moellendorffii, Oryza sativa, Aquilegia caerulea and Arabidopsis thaliana, and related non-WIP C2H2 zinc finger transcription factors from M. polymorpha and A. thaliana, AtSENSITIVE TO PROTON RHIZOTOXICITY 1 (AtSTOP1), MpSTOP1, AtJACKDAW (AtJKD) and MpINDETERMINATE(D)-DOMAIN 2 (MpiDD2), implementing the L-INS-i strategy. The alignment was manually trimmed to remove poorly-aligned regions (Fig. S1, Supplemental File 1). A maximum-likelihood phylogeny was estimated with PhyML 3.0 (Guindon et al., 2010), using the LG substitution model and NNI tree improvement. Branch support was estimated using the aLR-T SH-like method.

**Supplementary File 1**

Click here to Download Supplementary File 1
List of oligonucleotides used in this study:

Cloning of MpWIP:
MpWIP-F  tctctctctctctctctatc
MpWIP-R  tgtgtcaacgaatatctcagag

Generation of MpWIP promoter reporter construct:
proMpWIP1-F  aaatcactgactgcaattgaagg
proMpWIP1-R  acccgaagcttctgaacgtta
proMpWIP2-F  cgtgctcagacccccttc
proMpWIP2-R  caccatctgtcatgtgcctgc
proMpWIP3-F  gcccatcataacgcaccac
proMpWIP3-R  ttatttcaagctccgcgacg
proMpWIP4-F  gcagacattgattgaagtcgc
proMpWIP4-R  gggatgatggcaaggct
proMpWIPInFusion-F  aacgaaagctcgatccgtctagaccccagcttgaacgttaa
proMpWIPInFusion-R  ttcgagctcggtaacccggttaacgtctcagacccccttc

Generation of SRDX and VP16 fusions:
MpWIP-CDS-F  atgagctcaggtaccatgcc
MpWIP-SRDX-R  ctacgcaaagcccaagccggaatccctgcagttcagatccagtgcagtcgac
MpWIP-VP16-R  ctacagcatctcagactgaagtctctccagggcgctcgtctgcgtcggccgac

RT-PCR:
MpWIP-qRT-F  cgtggggctaatgagaaatg
MpWIP-qRT-R  atgcccatcgaagtcgaag
MpACT-qRT-F  aggcattcgttccacgag
MpACT-qRT-R  acatggtcgtctcctccagac
MpAPT-qRT-F  cgaaagcacaagagctacc
MpAPT-qRT-R  gtacccccggtsgccataag
MpWIP-CDS-RT-F  tgtcagcaagacctcaacc
MpWIP-SRDX-RT-R  cagtttcagatccagatcca
MpWIP-VP16-RT-R  tccaagtcgaagtcgttccagctcctcagacccccttc

Development 144: doi:10.1242/dev.144287: Supplementary information
SUPPLEMENTARY REFERENCES


