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 Table S2.
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.
Figure S4

Early stages of air pore complex development in proOsACT:amiR-MpWIP-3'UTRmpmiR160. 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 Table S1 for sequences of primers.

Constitutive expression of MpWIP

For constitutive expression of Mp\textit{WIP}, 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 \textit{pro OsACT:MpWIP} expression construct, an LR reaction was carried out between the entry vector and the plasmid \textit{proOsACT:GATEWAY:TERM-pMpGW207} (Breuninger et al., 2016).

Generation of \textit{proMpWIP:3xYFP-NLS}

To generate the \textit{proMpWIP:YFP-NLS} expression vector, 4.7 kb of sequence 5' to the start of the CDS of Mp\textit{WIP} was amplified in 4 overlapping segments using Phusion DNA polymerase with the primer pairs \textit{proMpWIP1-F} and \textit{proMpWIP1-R}; \textit{proMpWIP2-F} and \textit{proMpWIP2-R}; \textit{proMpWIP3-F} and \textit{proMpWIP3-R}; and \textit{proMpWIP4-F} and \textit{proMpWIP4-R}. These fragments were joined by overlap PCR using Phusion High-Fidelity DNA Polymerase (New England Biolabs) and primers \textit{proMpWIP4-F} and \textit{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 \textit{hpt} gene. The promoter was amplified with the primers \textit{proMpWIPInFusion-F} and \textit{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 SmaI (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 \textit{proMpWIP:GW:Term}. An LR reaction was carried out between this vector and the plasmid “\textit{NLS-3xYFP in pENTRY3c}” (Breuninger et al., 2016) to create an expression vector containing \textit{proMpWIP:3xYFP-NLS:Term}.

Constitutive expression of MpWIP-SRDX and MpWIP-VP16 fusion proteins

To generate a fusion between the EAR-motif repression domain (SRDX) and the C-terminus of Mp\textit{WIP}, the Mp\textit{WIP} CDS was amplified from the \textit{pro OsACT:MpWIP} expression vector with Phusion High-Fidelity DNA Polymerase and the primers \textit{MpWIP-CDS-F} and \textit{MpWIP-SRDX-R}. Mp\textit{WIP-SRDX-R} replaces the STOP codon of \textit{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 pDONR221 using BP Clonase II (Invitrogen) to create entry clones, which were each 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 tblastn algorithm. The most similar match
was At**NO 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(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.
### Table S1. List of oligonucleotides used in this study:

**Cloning of MpWIP:**

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<thead>
<tr>
<th>Oligonucleotide</th>
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<tr>
<td>MpWIP-F</td>
<td>tctctctctctctctctctatc</td>
</tr>
<tr>
<td>MpWIP-R</td>
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**Generation of MpWIP promoter reporter construct:**

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<th>Sequence</th>
</tr>
</thead>
<tbody>
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<tr>
<td>proMpWIP1-R</td>
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<td>proMpWIPInFusion-R</td>
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**Generation of SRDX and VP16 fusions:**

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<tr>
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<tr>
<td>MpWIP-VP16-R</td>
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**RT-PCR:**

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<tr>
<td>MpWIP-SRDX-RT-R</td>
<td>cagttcagatccagatcc</td>
</tr>
<tr>
<td>MpWIP-VP16-RT-R</td>
<td>tccagatcgagctgatcc</td>
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Table S2. An alignment of the WIP domain of MpWIP and WIP proteins in FASTA format

Click here to Download Table S2
SUPPLEMENTARY REFERENCES


