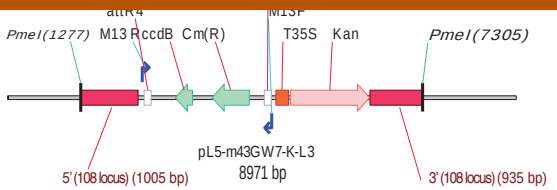
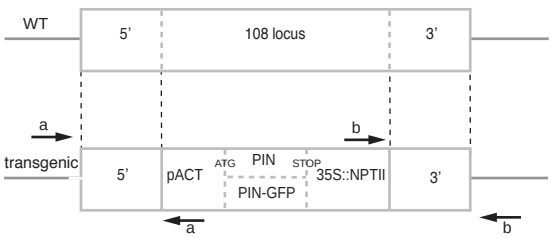
Suppl. Fig 1

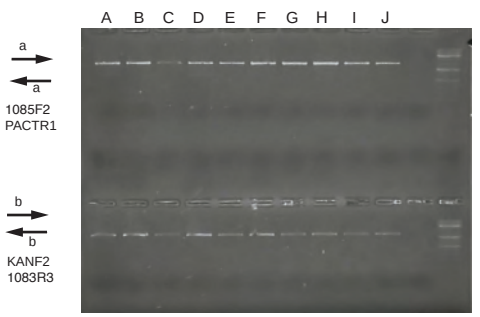


B

PpPINA AKKSMPPSAVM 552 **GFP** IKLIAVMTFRKLVWNPNTYS
PpPINB AKKSMPPSAVM 552 **GFP** IKLIAVMTFRKLVWNPNTYS
AtPIN1 QAKVMPPTSVM 461 **GFP** TRLILIMVWRKLIRNPNSYS
PpPIND KEVRKMGQPAV 163 **GFP** GSVAQRNSFSVANGESGTRE

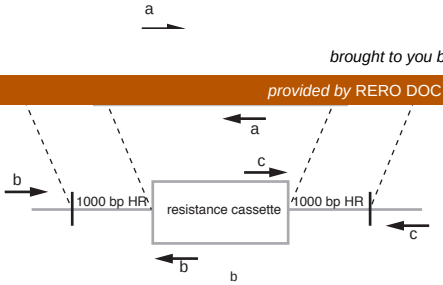
C

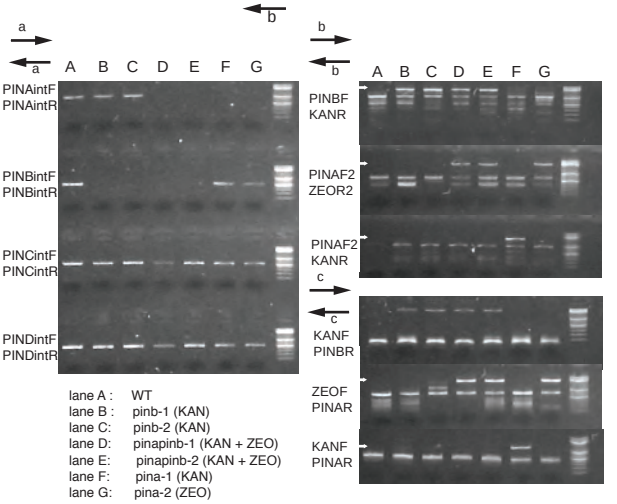




lane A : pACT::PpPINA
lane B : pACT::PpPINB
lane C: pACT::PpPINC-1
lane D: pACT::PpPINC -2
lane E: pACT::PpPIND
lane F: pACT::PpPINA-GFP
lane G: pACT::PpPINB-GFP
lane H: pACT::PpPIND-GFP
lane I: pACT::AtPIN1-1
lane J: pACT::AtPIN1-2

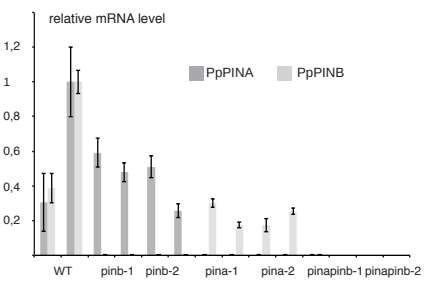
D



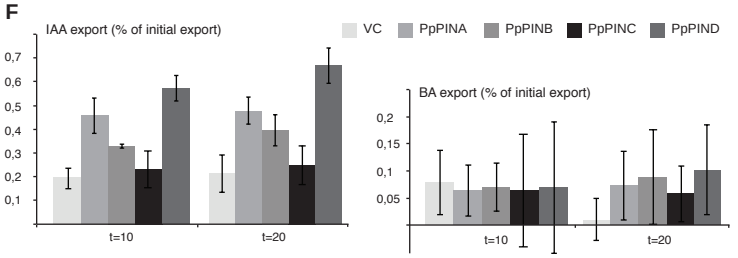


lane A : WT
lane B : pinb-1 (KAN)
lane C: pinb-2 (KAN)
lane D: pinapinb-1 (KAN + ZEO)
lane E: pinapinb-2 (KAN + ZEO)
lane F: pina-1 (KAN)
lane G: pina-2 (ZEO)

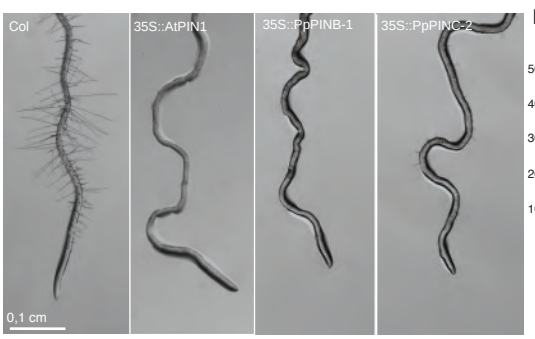
E



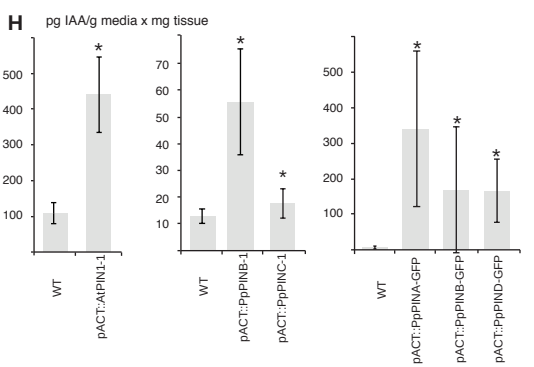
F



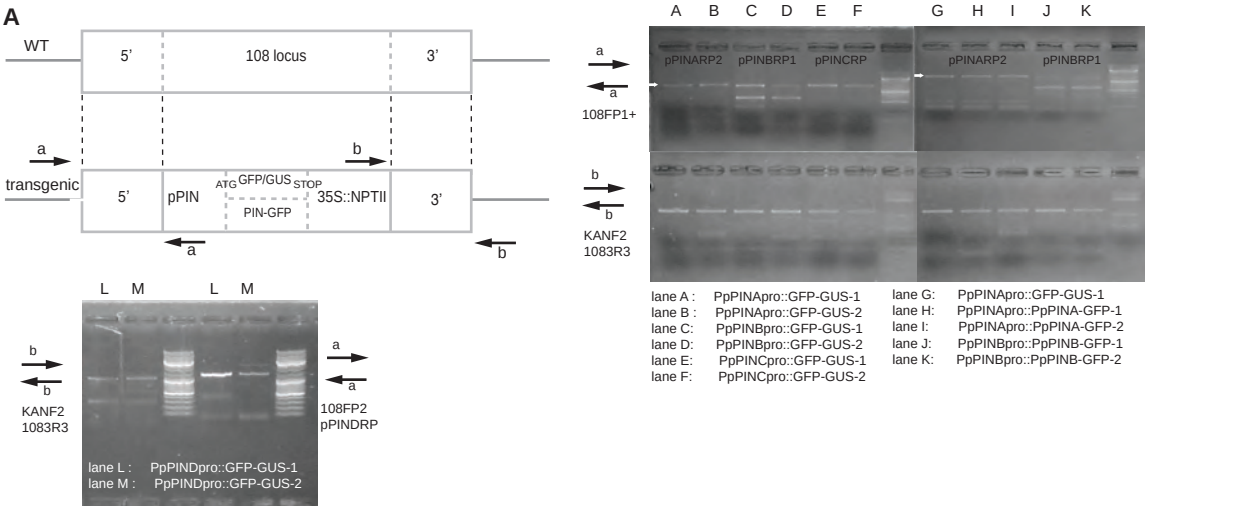
G

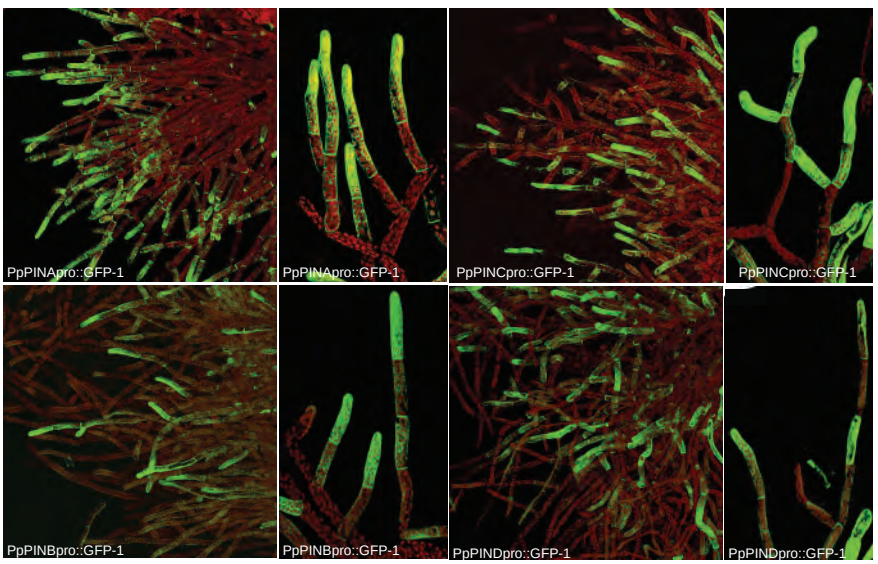


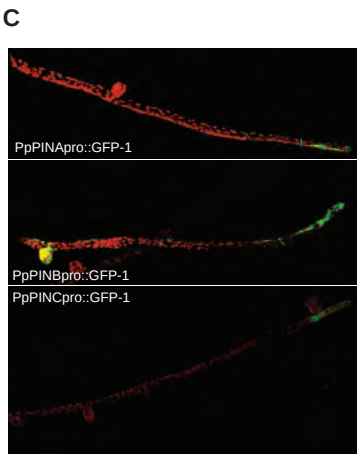
H

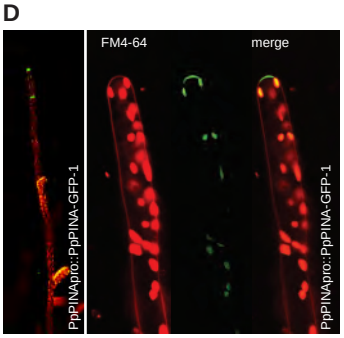


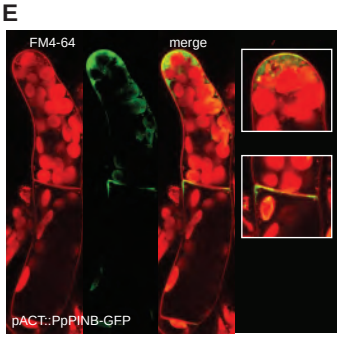
Suppl. Fig.2


A
WT
5'
108 locus
3'
a
b
transgenic
5'
pPIN
ATG GFP/GUS STOP
PIN-GFP
35S::NPTII
3'
a
b
A B C D E F
G H I J K
pPINARP2
pPINBRP1
pPINCRP
pPINARP2
pPINBRP1
a
a
108FP1+
b
b
KANF2
1083R3
lane A : PpPINApro::GFP-GUS-1
lane B : PpPINApro::GFP-GUS-2
lane C: PpPINBpro::GFP-GUS-1
lane D: PpPINBpro::GFP-GUS-2
lane E: PpPINCpro::GFP-GUS-1
lane F: PpPINCpro::GFP-GUS-2
lane G: PpPINApro::GFP-GUS-1
lane H: PpPINApro::PpPINA-GFP-1
lane I: PpPINApro::PpPINA-GFP-2
lane J: PpPINBpro::PpPINB-GFP-1
lane K: PpPINBpro::PpPINB-GFP-2
L M L M
b
b
KANF2
1083R3
a
a
108FP2
pPINDRP
lane L : PpPINDpro::GFP-GUS-1
lane M : PpPINDpro::GFP-GUS-2


B
PpPINApro::GFP-1
PpPINApro::GFP-1
PpPINCpro::GFP-1
PpPINCpro::GFP-1
PpPINBpro::GFP-1
PpPINBpro::GFP-1
PpPINDpro::GFP-1
PpPINDpro::GFP-1

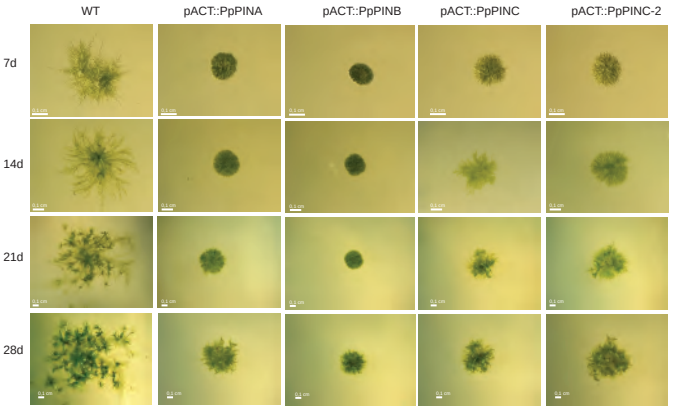

C
PpPINApro::GFP-1
PpPINBpro::GFP-1
PpPINCpro::GFP-1


D
FM4-64
merge
PpPINApro::PpPINA-GFP-1
PpPINApro::PpPINA-GFP-1

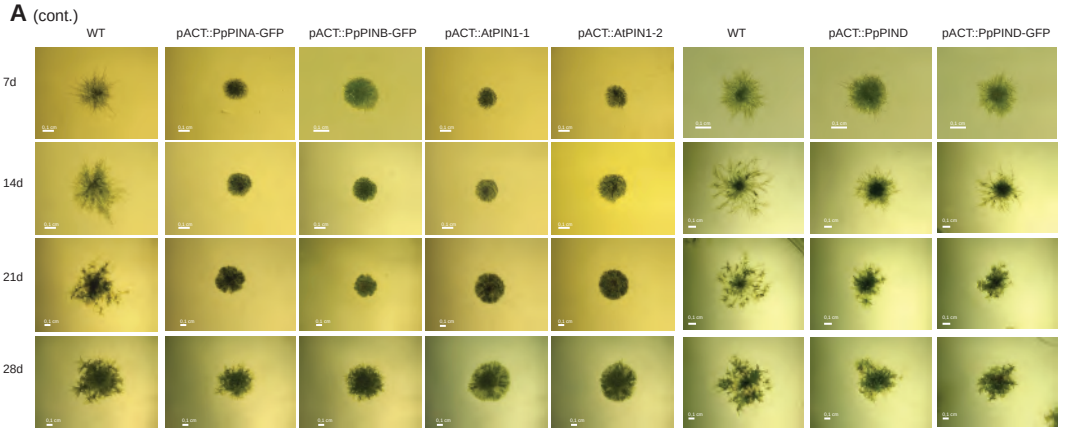

E
FM4-64
merge
pACT::PpPINB-GFP

Suppl. Fig.3

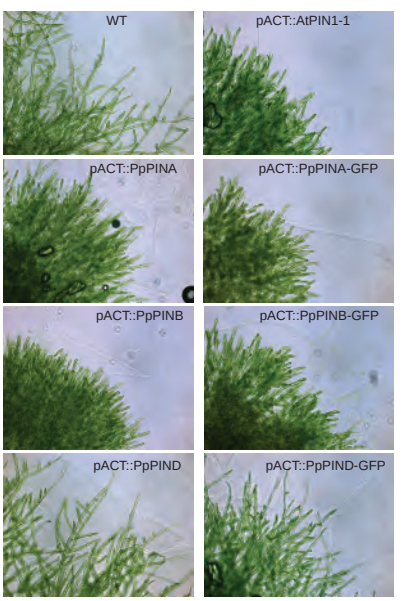
A



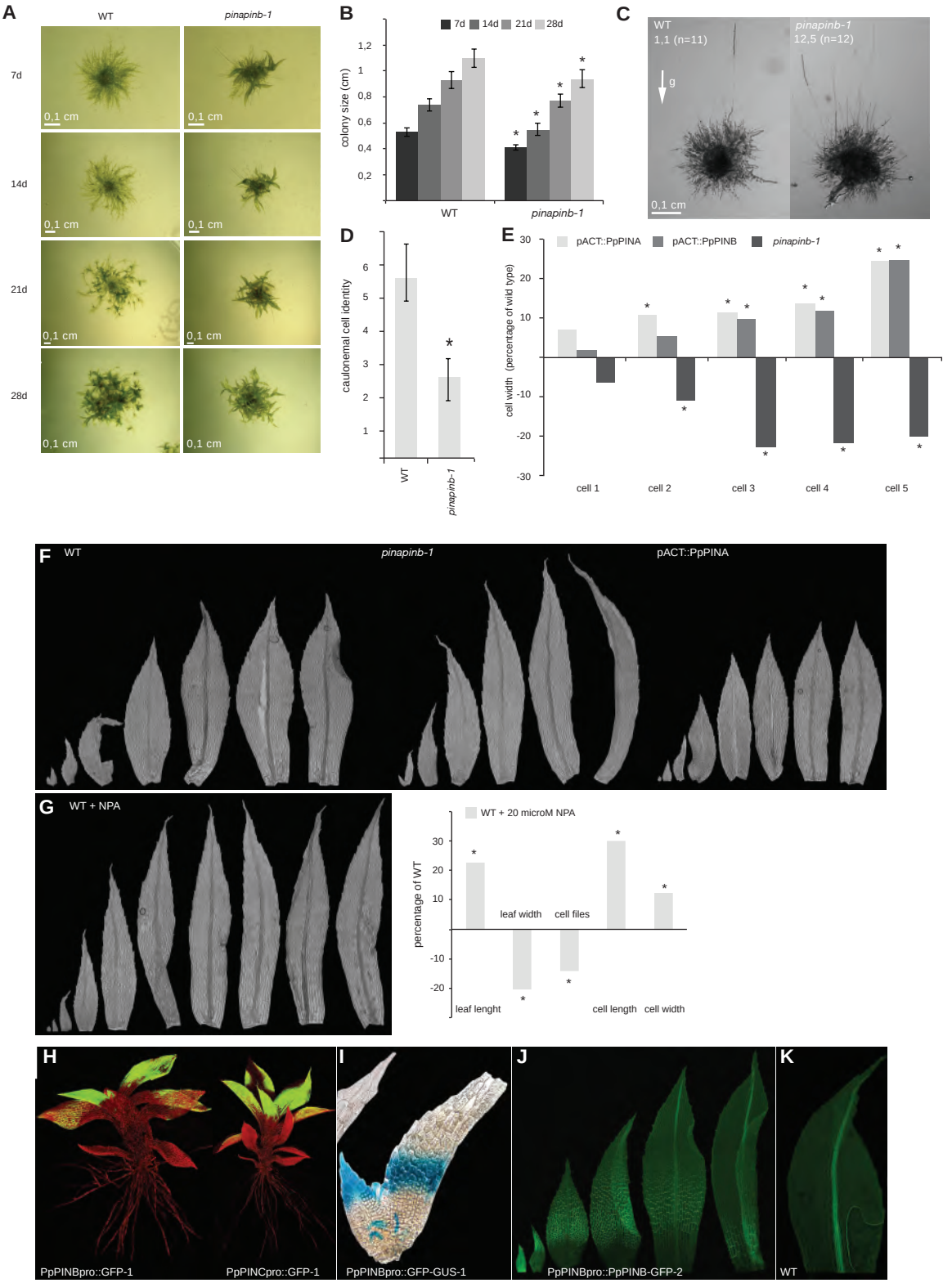
A (cont.)



B



Suppl. Fig.4


A
WT
pinapinb-1
7d
14d
21d
28d
0,1 cm
B
7d 14d 21d 28d
colony size (cm)
1,2
1
0,8
0,6
0,4
0,2
WT
pinapinb-1
C
WT
1,1 (n=11)
pinapinb-1
12,5 (n=12)
g
0,1 cm
D
caulonemal cell identity
6
5
4
3
2
1
WT
pinapinb-1
E
pACT::PpPINA
pACT::PpPINB
pinapinb-1
cell width (percentage of wild type)
30
20
10
-10
-20
-30
cell 1
cell 2
cell 3
cell 4
cell 5
F
WT
pinapinb-1
pACT::PpPINA
G
WT + NPA
WT + 20 microM NPA
percentage of WT
30
20
10
-10
-20
leaf width
cell files
leaf lenght
cell length
cell width
H
PpPINBpro::GFP-1
PpPINCpro::GFP-1
I
PpPINBpro::GFP-GUS-1
J
PpPINBpro::PpPINB-GFP-2
K
WT

Figures S1-S4

Figure S1. Vector map, translational fusions to eGFP, generation of PIN OE and knockout lines in *P. patens*, auxin transport assays of PIN proteins in *N. benthamiana* protoplasts, *Arabidopsis* root hair assays and *P. patens* auxin export assays (supplement to Figure 1)

(**A**). Vector map of the gateway multisite destination vector pL5-m34GW7-K-L3 used to target the *P. patens* 108 silent locus by homologous recombination (*36*). (**B**). Protein alignment showing the position where eGFP was inserted to generate the translational fusions. The number indicates the amino acid position in the PIN protein sequence. (**C**). Principal construct overview and PCR confirmation of transgenic *P. patens* lines overexpressing different PIN proteins, with or without the eGFP-tag. (**D**). Principal construct overview and PCR confirmation of *P. patens PpPINA* and *PpPINB* single and double KO lines. (**E**). qPCR showing transcriptional activity of *PpPINA* and *PpPINB* in WT, single and double KO lines. (**F**) Auxin transport assays in mesophyll protoplasts from transiently transfected *N. benthamiana* leaves indicate that PpPINs enhance export of IAA and not BA over time. Presented are average values of IAA and BA export measurements from at least 3 independent protoplast infiltrations at two different timepoints (in minutes). VC (empty silencing *Agrobacterium*) was used as control. (**G**). Overexpression of long PINs from both *Arabidopsis* (*AtPIN1*) and *P. patens* (*PpPINB* and *PpPINC*) strongly inhibits root hair development in *Arabidopsis*. Data are presented as mean +- s.d. All primers used in this figure can be found in Table S1. **(H**). Transgenic *P. patens* lines overexpressing long PINs from

Arabidopsis (*AtPIN1*) and *P. patens* PpPINs (*PpPINB* and *PpPINC; PpPINA-GFP, PpPINBGFP* and *PpPIND-GFP*) enhance IAA export into the medium. Free IAA in liquid media per mg moss tissue is shown. Data are presented as mean +- s.d.; * P<0.05 (by Student's t-test).

Figure S2. Generation of *PpPIN* transcriptional and translational fusions and expression and localization of PIN proteins during protonemal development (supplement to Figure 2)

(**A**). Principal construct overview and PCR confirmation of transcriptional and translational reporter *P. patens* lines under endogenous *PpPIN* promoters. All primers used in this figure can be found in Table S1. (**B**). Transcriptional reporter lines show activity of the long *PpPINA-D* genes during *P. patens* chloronemal development. Expression of long *PpPINA-C* gradually increases towards the tip of the chloronemal filaments. Transcriptional reporter line of the short *PpPIND* proteins shows a less pronounced graded expression in chloronemal filaments. (**C**). Transcriptional reporter lines show activity of the long PpPIN genes during *P. patens* caulonemal development. *PpPINA-C* and promoters show an increasing activity towards the tip in caulonemal filaments. (**D**). The PpPINA-GFP-1 fusion protein expressed by PpPINAs native promoter co-localizes with the fluorescent PM-marker FM4-64 at the apical membrane of the tip cell in a caulonemal filament. (**E**) Overexpression of translational fusion of the long PpPINB to GFP shows co-localization with FM4-64 at the distal cell side towards the filament tip. All pictures are taken using a Carl Zeiss LSM 710 confocal microscope. Autofluorescence of chloroplasts is obvious in both the green and red channel.

Figure S3. Overexpression of long and short PpPINs (with and without eGFP) and AtPIN1 in

P. patens (supplement to Figure 3)

(**A**). *P. patens* colonies of all different OE genotypes (with and without eGFP) and WT were followed over time during four weeks. Pictures were taken with a binocular microscope. In general, long PpPIN OE (with and without the eGFP tag) and AtPIN1 OE lines show a significant reduced colony proliferation compared to WT. Colony growth of the short PpPIND OE line (with and without tag) is very similar to WT and only slightly reduced in size. While OE of long PpPIN (with and without tag) and AtPIN1 delays the appearance of gametophores, OE of short PpPIND (with and without tag) does not change the timing and amount of gametophores on the colonies. Numerical data can be found in Table S2 (**B**). Detailed pictures of colony edges of selected OE genotypes; OE of long PpPIN (with and without tag) and AtPIN1 reduces the growth of protruding caulonemal filaments, while OE of short PpPIND (with and without tag) behaves like WT.

Figure S4. Detailed analyses of protonemal development in *pinapinb* KO and PIN OE lines, expression and localization of long PpPINs during gametophore development and detailed analyses of gametophore development in *pinapinb* KO and PIN OE lines (supplement to Figure 3 and 4)

(**A**). Colonies of *pinapinb* KO and WT followed during four weeks. (**B**). The *pinapinb* mutant line produces consistently smaller colonies. (**C**). Dark-grown colonies of a dKO line produce more caulonemal filaments than WT in the same timeframe. Numbers indicate the average number of caulonemal filaments growing out from the colony. The white arrow indicates the

gravity vector. (**D**). Regenerating chloronemal filaments from protoplasts show an earlier transition to caulonemal cell identity in a *pinapinb* mutant line. Data is presented as the first cell in the regenerating filament that shows caulonemal cell identity. Data are represented as mean +- s.d.; * P<0.05 (by Student's t-test). (**E**). OE and dKO of long PIN proteins have an opposite effect on the cell width in regenerating protonemal filaments. While OE increases, dKO of long PIN proteins reduces cell width significantly. (**F**). Leaf developmental series of WT (leaf P1-P8), *pinapinb* mutant (leaf P2-P8) and PpPINA OE lines (leaf P1-P8) show altered leaf development. *Pinapinb* mutant leaves are narrower and longer, while PpPINA OE leaves are narrower and shorter compared to WT leaves. (**G**). Leaf developmental series of WT colonies grown on 20 microM NPA (leaf P1-P10) show altered leaf development, with narrower and longer leaves, similar to *pinapinb* dKO lines. They also have a reduced number of cell files, pointing towards a reduced cell division. Cells in the leaves are longer and wider, suggesting stimulated elongation of cells, when treated with 20 microM NPA. Data is presented as the percentage of increase or decrease compared to WT. Data are represented as mean +- s.d.; * P<0.05 (by Student's t-test). (**H**). The *PpPINC* and *PpPINB* promoters are highly active in developing leaves of the gametophytic shoots. (**I**). GUS staining in leaves of *PpPINB* transcriptional reporter line reveals a strong *PpPIN* expression domain at the tip of young leaves that gradually moves towards the base of the leaves as well as expression in the axillary hairs. (**J**). Translational reporter line of *PpPINB* shows a basipetal wave of PIN expression during leaf development (from leaf P3 to leaf P8). **(K).** WT leaf showing strong autofluorescence in the midrib cells of fully developed leaves.

Table S1. Overview of primers used in this paper.

Table S2. Detailed comparison of developmental phenotypes and transgene expression of different genotypes expressing PIN proteins, with or without tag, under the actin promoter.

(A-B) Colony size (in square mm) and number of gametophores is followed during four weeks of growth. In general, long PpPIN OE (A-C, with and without the eGFP tag) and AtPIN1 OE lines show a significant reduced colony proliferation compared to WT. Colony growth of the short PpPIND OE line (with and without tag) is very similar to WT and only slightly reduced in size. While OE of long PpPIN (with and without tag) and AtPIN1 delays the appearance and decreases the total amount of gametophores, OE of short PpPIND (with and without tag) does not change the timing and amount of gametophores on the colonies. Data are presented as mean with s.d. in between brackets. Number is highlighted when significantly different from WT ($p<0.005$, t-test). **(C)** Relative transgene expression by qPCR (both PIN and resistance cassette) is shown in the *P. patens* OE lines.

Supplementary Table 1

KNOCKOUT		
pDONR P4-P1R	PINAHRattB4F	GGGGACAACTTTGTATAGAAAAGTTGCCTGTATCCTGTTCACACTGCG
	PINAHRattB1R	GGGGACTGCTTTTTTGTACAAACTTGCTTTGAGAACGTTTGTCCGGC
	PINBHRattB4F	GGGGACAACTTTGTATAGAAAAGTTGCCATATGTTGGGTGCGTCATTCT
	PINBHRattB1R	GGGGACTGCTTTTTTGTACAAACTTGCTTTGACAGAGGTTTAAGTACTG
pDONR221	ZeoattB1F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCCCTTTCAGAAAGAATGCTAACC
	ZeoattB2R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCCGTCACCGGTGTGAGGGA
	KanattB1F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCCCATGGAGTCAAAGATTCAAAT
	KanattB2R	GGGGACCACTTTGTACAAGAAAGCTGGGTCATTCGAGCTCGGTACCCCTG
pDONR P2R-P3	PINAattB2F	GGGGACAGCTTTCTTGTACAAAGTGGCCTTGGCTGGCAAGTGCTATTTT
	PINAattB3R	GGGGACAACTTTGTATAATAAAGTTGCAAACTGGCCATGTCGGTCAA
	PINBattB2F	GGGGACAGCTTTCTTGTACAAAGTGGCCGTGACTGGAAATGCCGCTTT
	PINBattB3R	GGGGACAACTTTGTATAATAAAGTTGCTTACCGAACATTGCGAGCATG
AMPLIFICATION	PINAKOF	TGTATCCTGTTCACACTGCG
	PINAKOR	AAACTGGCCATGTCGGTCAA
	PINBKOF	ATATGTTGGGTGCGTCATTCT
	PINBKOR	TTACCGAACATTGCGAGCATG
GENOTYPING	PINAintF	GCCTCCCACTTGCATGAATA
	PINAintR	ATTGTGGAAAACCCATGCTC
	PINBintF	CATCTCCCACAGGAGTTCGT
	PINBintR	TCAGTTGGTGATGGCAAGAA
	PINCintF	GGCCTCTCTGAACAAAGCTG
	PINCintR	CGAATTTTTAATGCTCTTATGTTGAA
	PINDintF	TCCCTGTCTGTTTCGACTACAA
	PINDintF	ATTCACGCAGGCTCAAAAGT
	PINAF2	CTGTGCATTTGCTGTGAGGT
	ZEOR2	GGGGCTTATGCGGATTATTT
	ZEOF	GAACTCGCCGTAAAGACTGG
	PINAR	GAATGTGCTAGCAGGCGATT
	KANR	CATGGGTCACGACGAGATCCT
	PINBF	ATATGTTGGGTGCGTCATTCT
	KANF	TCGCCTTCTTGACGAGTTCT
	PINBR	CCAAAGCAAACCAGTCAACA
	PINAF2	CTGTGCATTTGCTGTGAGGT
	KANR	CATGGGTCACGACGAGATCCT
	KANF	TCGCCTTCTTGACGAGTTCT
qPCR		
	PpPINAFPqPCR	AGTGCGCATGCTTGTACATC
	PpPINARPqPCR	CCAAAGCTGAAGTCCTCTCG
	PINBqPCRFb	GTTGCAAATAGGTGGCATTTC
	PINBqPCRRb	ATCCCAAGTCCTGCATCTGA
	ACT3qPCRFb	GAATGGTCAAGGCTGGTTTC
	ACT3qPCRRb	TACCGACCATCACACCAGT
	PpL21FqPCR	GTACTCGAGAAGCCAGACTTCCTAC
	PpL21RqPCR	TCAATCTTCTTAGCATCACGGTACT
	PpPINCFPqPCR	AGAAGTGCGTATGCTTGTGC
	PpPINCRPqPCR	AAGTTCTGGGGTTCGCTTCT
	PpPINDFPqPCR	ACCGCCTAATTGCTGCATAC
	PpPINDRPqPCR	CACAATCCAACGAGAACGATT
	KANF	ATCCATCATGGCTGATGCAATGCG
	KANR	GATGTTTCGCTTGGTGGTCGAAT
	PIN1-Fw	TACTCCGAGACCTTCCAACTA
	PIN1-Rv	TCCACCGCCACCACTTCC
	QEIF4A_FW	ACGGAGACATGGACCAGAAC
	QEIF4A_REV	GCTGAGTTGGGAGATCGAAG
OVEREXPRESSION		
pDONR P4-P1R	proACT1attB4F	GGGGACAACTTTGTATAGAAAAGTTGTTTCGAGGTCATTCATATGCTTG
	proACT1attB1rR	GGGGACTGCTTTTTTGTACAAACTTGTGATATCCTCGGCGTCAGC
pDONR221	ATTB1PPPINAFP	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGATTAACGGCCATGACATA
	ATTB2PpPINAstopRP	GGGGACCACTTTGTACAAGAAAGCTGGGTTtcacagaccaagtaatatgta
	ATTB1PPPINBFP	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGATTAACGGGCATGACATT
	ATTB2PpPINBstopRP	GGGGACCACTTTGTACAAGAAAGCTGGGTTtcacagaccaagtaatatgta
	ATB1PPPINCFP2	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGATTACGGGTCATGACATG
	ATTB2PpPINCstopRP	GGGGACCACTTTGTACAAGAAAGCTGGGTTtcagaggcccagtaggatgta
	ATTB1PPPINDFP	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGTTGACAGGTGGTCAGTTT
	ATTB2PpPINDstopRP	GGGGACCACTTTGTACAAGAAAGCTGGGTTtcagagttcaagtagtgtgta
	attB1FPIN1F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGATTACGGCGGCGGACTT
	attB2RPIN1R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATAGACCCAAGAGAATGTAG
GENOTYPING	Pp5locus108FP2	TCAACCTTGTATTACATCCACGA
	pACTgenotRP1	TCACTTTGGGCCACCTTTTA
	KANcassforwardgenotyp2	GCGCGGTGTCATCTATGTTA
	108locus3Rgenotyp3	TCATCCATCCTTTCTTCATGC
	35SgenotypR	CTTTCTCTGTGTTCTTGATGCAG

TRANSCR./TRANSL. FUSIONS		
pDONR P4-P1R	PpPINApromFP	GGGGACAACTTTGTATAGAAAAGTTGTTCGCAGAATCTGTCGCCTTTCC
	PpPINApromRP	GGGGACTGCTTTTTTGTACAAACTTGTTTTGAGAACGTTTGTCCGGCC
	PpPINBpromFP	GGGGACAACTTTGTATAGAAAAGTTGTTTGGATAATCGTAACTATAATA
	PpPINBpromRP	GGGGACTGCTTTTTTGTACAAACTTGTTTTGACAGAGGTTTAAGTACT
	PpPINCpromFP	GGGGACAACTTTGTATAGAAAAGTTGTTTCCTTTGTGTGGAGTAGAGTT
	PpPINCpromRP	GGGGACTGCTTTTTTGTACAAACTTGTCTTGACGATCTGTCGACGTGC
	PpPINDpromFP	GGGGACAACTTTGTATAGAAAAGTTGTTGCGAGGGACGGGGTTTCCTTC
	PpPINDpromRP	GGGGACTGCTTTTTTGTACAAACTTGTGACGATAACGTTGATTGTCCT
overlapping PCR	PINARP1gfp2loop	tcctcgcccttgctcaccatCATCACAGCAGAAGGTGGCA
	PINAFP2gfp2loop	gcatggacgagctgtacaagATTAAGCTAATTGCGGTCATG
	GFPFPpina2loop	TGCCACCTTCTGCTGTGATGatggtgagcaagggcgagga
	GFPRPpina2loop	CATGACCGCAATTAGCTTAATcttgtacagctcgtccatgc
	PINBRP1gfp2	tcctcgcccttgctcaccatCATCACTGCCGAAGGTGGCA
	PINBFP2gfp2	gcatggacgagctgtacaagATCAAGCTCATCGCTGTCATG
	GFPFPpinb2	TGCCACCTTCGGCAGTGATGatggtgagcaagggcgagga
	GFPRPpinb2	CATGACAGCGATGAGCTTGATcttgtacagctcgtccatgc
	PINDRP1gfp2	tcctcgcccttgctcaccatCACGGCAGGCTGACCCATTT
	PINDFP2gfp2	gcatggacgagctgtacaagGGTTCCGTCGCACAACGAAA
	GFPFPpind2	AAATGGGTCAGCCTGCCGTGatggtgagcaagggcgagga
	GFPRPpind2	TTTCGTTGTGCGACGGAACCcttgtacagctcgtccatgc

Supplementary Table 2

GENOTYPE	size (square mm)				# gametophores				relative transgene expression	
	1 WEEK	2 WEEKS	3 WEEKS	4WEEKS	1 WEEK	2 WEEKS	3 WEEKS	4WEEKS	PIN	nptII
WT	6,61 (0,56)	21,95 (2,85)	35,63 (4,62)	60,90 (8,23)	0,00	5,92 (1,34)	11,54 (1,76)	20,5 (3,30)		
pACT::PpPINA	5,42 (0,47)	10,86 (0,55)	20,91 (2,10)	37,96 (2,65)	0,00	0,17 (0,39)	3,23 (1,09)	17,42 (2,84)	1x	1x
pACT::PpPINA-gfp	3,98 (0,43)	6,82 (0,81)	11,21 (1,19)	20,51 (1,57)	0,00	0,00	1,43 (0,98)	9 (2,34)	6,8x	3,4x
pACT::PpPINB	2,89 (0,39)	4,10 (0,61)	7,91 (0,99)	16,97 (2,79)	0,00	0,08 (0,28)	0,46 (0,78)	6,17 (2,59)	1x	1x
pACT::PpPINB-gfp	3,50 (0,39)	6,17 (0,98)	12,94 (1,15)	28,30 (1,87)	0,00	1,08 (0,9)	5,75 (2,26)	14 (1,95)	2,2x	2,5x
pACT::PpPINC	5,26 (0,54)	10,95 (1,07)	18,39 (1,92)	33,64 (3,06)	0,00	3,1 (1,37)	7,82 (1,08)	14,73 (2,41)	1x	1x
pACT::PpPINC-2	5,90 (0,31)	12,75 (1,09)	24,15 (2,44)	44,37 (2,88)	0,00	1,6 (1,07)	8,3 (2,36)	18,8 (2,44)	1,1x	1,35x
WT	7,12 (1,55)	15,23 (1,55)	33,25 (3,92)	52,24 (8,39)	0,00	2,64 (1,50)	9,89 (2,32)	16,9 (2,92)		
pACT::PpPIND	4,79 (0,44)	13,39 (0,96)	21,84 (2,87)	41,32 (9,51)	0,00	2,45 (0,93)	8,5 (1,24)	15,09 (2,47)	1x	1x
pACT::PpPIND-gfp	5,11 (0,35)	15,37 (2,34)	20,87 (4,62)	36,77 (7,57)	0,00	4,25 (1,66)	11,33 (1,72)	17,58 (2,42)	4,9x	4,1x
WT	7,44 (1,05)	23,91 (2,77)	37,03 (4,62)	59,22 (7,34)	0,13 (0,35)	4,13 (1,46)	12,00 (2,58)	20,14 (2,73)		
pACT::AtPIN1-1	4,21 (0,78)	12,62 (2,17)	24,98 (3,39)	41,28 (5,04)	0,00	0,00	1,13 (1,13)	6,71 (3,45)	4,7x	1,4x
pACT::AtPIN1-2	3,95 (0,21)	9,72 (1,57)	18,74 (2,29)	30,36 (4,28)	0,00	0,00	1,13 (1,13)	5,14 (2,27)	1x	1x

significant when p<0,005 (t-test)
(standard deviation)

Supplemental Experimental Procedures

Phylogenetic reconstruction

We used the protein matrix from [S1], but kept only *P. patens*, *Selaginella moelendorfii* and *Arabidopsis* PIN proteins and the outgroup sequence from *Trichomonas vaginalis* and *Klebsormidium flaccidum*. Phyml 3.0 was used to reconstruct a maximum likelihood tree. ML and NJ bootstrap values were calculated using Phyml3.0 and the Phylogeny.fr platform [S2-4].

Auxin transport assays in mesophyll cells from *N. benthamiana*

The coding sequences from the four PpPIN proteins were cloned with stop codon into pDONR221 and subsequently into the gateway destination vector pH2GW7 (for primers, see Suppl. Table 1). This vector was then transformed in the *Agrobacterium* strain C58C1 using electroporation. After agroinfiltration of tobacco leaves, protoplast preparation and transport assays were performed as in [S5]. Relative IAA/NAA export is calculated from effluxed radioactivity as follows: ((radioactivity in the medium at time t) - (radioactivity in the medium at time t=0)) * (100%)/ (radioactivity in the medium at t=0).

Root hair assay in *Arabidopsis*

The pDONR221 vectors with the coding sequences including the STOP codon for the four PpPIN proteins were recombined into the gateway destination vector pB7WG2. This vector was used to transform *Arabidopsis*, Columbia ecotype, using the *Agrobacterium* strain C58C1 (Fig. S2B). Transformed plants were selected on BASTA-containing plates (15 mg/l). T3-seedlings were grown vertically in Petri dishes on 0.8% agar 0.5× Murashige and Skoog (MS)

medium containing 1% sucrose, pH 5.9, at 18°C and under a long day light regime. Pictures of the primary root were taken after 8 days of growth with a stereomicroscope.

Cultivation of *P. patens* material

Protonemal tissue was subcultured several times with a minimum of 7 days in between using the ULTRA-TURRAX Tube Drive worksystem (IKA). Subculturing was on cellophane-covered plates with BCD medium [S6], supplemented with 5 mM ammonium tartrate and 0,8% agar. Tissue was grown at 24°C in a long day light regime. Light intensity in the growth chamber is 55 micromoles per m^{-2} s^{-}.

Vector construction

A Gateway multisite destination vector was developed for homologous recombination in *P. patens*. The 108 locus was used as a target of recombination *(Fig. S1A)* [S7]. A fragment of 1005 and 935 bp respectively from the first half (5') and second half (3') of the 108 locus were amplified and cloned in pGEM7ZF (Promega) to generate pL5-L3. A *PmeI* restriction site was included into the primers in order to facilitate removal of the vector backbone from the transgene construct. The Kanamycin marker cassette (nptII coding sequence controlled by a nos promoter and a nos terminator) was cloned between the 108 locus 3' and 5' fragments to obtain pL5-K-L3. A multisite Gateway destination cassette (attR4-ccdB-attR3- Invitrogen) fused at the attR3 region to the CaMV35S terminator was cloned between the 108 locus 5' fragment and the Kanamycin selectable marker to generate a pL5-m34GW7-K-L3 moss destination vector. After recombination, the destination vector is digested with the *PmeI* restriction enzyme and *the linearized DNA was transformed into P. patens protoplasts.*

***P. patens* OE transgenic lines**

The actin promoter from rice was cloned into pDONR P4-P1R [S8] and the coding sequence from AtPIN1 (including stop codon) was cloned into pDONR221 (for primers, see Table S1). All coding sequences from the four *PpPIN*s and *AtPIN1* in pDONR221 (including stop codon) were fused together with pACT through two Gateway intermediary vectors (pXb2-m43GW and pDONRP4P3) into the final destination vector (pL5-m43GW7-L3) to generate an overexpression construct (Fig. S1C-D).

Transcriptional fusions with GFP-GUS

A 2kb fragment upstream of the start of each PpPIN coding sequence was amplified and was cloned into pDONR P4-P1R (for primers, see Table S1). This was recombined, together with pEN-L1-F-L2 and pEN-R2-S-L3, into pL5-m43GW7-L3 to generate transcriptional reporter lines of each *PpPIN* gene (Fig. S3A).

Translational fusions with GFP

To generate translational fusions to GFP, overlapping PCR using the *PpPIN* genomic fragments and eGFP was used (Fig. S1B, for primers, see Tabe S1). The fusion fragment was cloned into pDONR221. These vectors were fused to the endogenous *PIN* promoters or to the actin promoter from rice, and recombined into pL5-m43GW7-L3 to generate translational fusions for each PIN protein (Fig. S1C-D, S3A)

Knockout construction

The design for knockout of both *PpPINA* and *PpPINB* is visualised in Figure S1F. A 1000 bp fragment upstream of the start and downstream of the stop were respectively cloned into pDONR P4-P1R and pDONR P2R-P3. The resistance cassettes for nptII from plasmid pMT164 [S6] and for zeocine from pCMAK1 [S9] were cloned into pDONR221 (for primers,

see Table S1). These vectors were then recombined into pXb2-m43GW. The full sequence (homologous recombination sequences and resistance cassette) was then amplified by PCR using iProof (Biorad) and several PCR-reactions were precipitated (for primers, see Table S1). The precipitated DNA was then used to transform *P. patens* protoplasts *(Fig. S1E-F)*. We attempted to generate a triple knockout line, including the long PpPINC, but failed to recover any triple mutant colonies. As the regeneration capacity of the pinapinb mutant lines are reduced compared to wild-type, we believe that triple mutant protoplasts simply cannot regenerate.

P. patens phenotyping

For phenotypic analysis of individual colonies, small pieces of fresh protonemal tissue were inoculated on BCD medium without cellophane and without ammonium tartrate in standard growth conditions. Colonies were photographed every week using a stereomicroscope. For protoplast regeneration, protoplasts were isolated similar to the transformation protocol. After 5 days on regeneration plates, protoplasts were transferred to BCD plates and grown under standard conditions. Pictures were taken with the Axio Imager microscope from Zeiss. To analyse gametophores, gametophores from same-aged colonies were harvested and dissected. Pictures were taken with a stereomicroscope (entire gametophores), or a Leica DMI4000B inverted microscope for close-up pictures of leaves.

GUS Staining and Fluorescence Microscopy

For histochemical staining, tissue was incubated in GUS staining solution [S10] over night at room temperature followed by de-staining in 70% ethanol before microscopic analysis. For fluorescence microscopy, dissected plant material was analyzed using a Leica DMI4000B inverted microscope or a Carl Zeiss LSM710 confocal microscope.

RNA and DNA isolation

Fresh protonemal tissue from *P. patens* and 2-week old seedlings from *Arabidopsis* were snap frozen in liquid nitrogen. Total RNA was extracted with Trizol (LifeTechnologies), followed by purification with RNeasy kit (Qiagen). DNA contaminants were removed using TURBO DNAse free-kit (Ambion). Superscript III (Invitrogen) was then used to prepare cDNA. Quantitative RT-PCR was performed on a LightCycler 480 Real-Time PCR System (Roche Diagnostics) with SYBR Green I Master Reagents (Roche Diagnostics) (for qPCR primers, see Table S1).

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