Supporting Information

Regulation of *MIR165/166* by Class II and Class III homeodomain leucine zipper proteins establishes leaf polarity

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Supplemental Materials and Methods

Plant material and treatments

Arabidopsis thaliana (L.) Heyhn plants were in Columbia-0 (Col-0) background. *HD-ZIPII* double (*hat3-1 athb4-1*) and triple (*hat3-1 athb4-1 athb2-1*) mutants, and *rev-10d hat3-1 athb4-1* triple mutant, were generated through genetic crosses, and identified as previously described (1-4). The reporter lines used for live imaging analysis were generated into *hat3 athb4* and *hat3 athb4 athb2*, which are segregating for the *hat3* mutation, and wild type background. *hat3/+ athb4/athb4* or *athb4/athb4* backgrounds, which do not show any phenotype, were used as control plants. The reporter lines generated into *hat3 athb4* background were: *pMIR165a::mTagBFP-ER*, *pMIR166a::GFP-ER*, *pUBQ10::REV-mEos2FP-ER* (miR165/166 biosensor), *pUBQ10::REVi-mEos2FP-ER* (miR165/166 insensitive biosensor), *pREV::REV-2xYPET* combined with *pPIN::PIN1-GFP*, *pREV::REVr-2xVENUS*, *pREV::REVr-2xVENUS* combined with *pMIR165a::mTagBFP-ER*, and *pML1::GR-LhG4* combined with *p6xOp::REVr-2xVENUS* and *pMIR165a::mTagBFP-ER*. The reporter lines generated into *hat3 athb4 athb2* background, which is segregating for the *hat3* mutation, were: *pREV::REVr-2xVENUS* and *pREV::REV-2xVENUS*. For confocal live imaging, *hat3/+ athb4/athb4* or *athb4/athb4* backgrounds, which do not show any phenotype, were used as control plants. The reporter lines generated exclusively in wild type background were: *pHAT3::VENUS-HAT3* and *pATHB4::VENUS-ATHB4*, both combined with *pMIR165a::GFP-ER*, *pUBQ10::GR-LhG4* combined with *p6xOp::MIR165a* and *pMIR166a::GFP-ER*, *pUBQ10::GR-LhG4* combined with *p6xOp::MIR165a*, *pMIR165a::GFP-ER* and *pHAT3::VENUS-HAT3* or *pATHB4::VENUS-ATHB4*, and *pML1::GR-LhG4* combined with *p6xOp::REVr-2xVENUS, pMIR165a::GFP-ER* and *pMIR165a(-cis)::mTagBFP-ER*. For seed

production and crosses, plants were grown in a soil/vermiculite/perlite mixture. For live imaging analysis, plants were grown in Gamborg's medium (GM) plates supplemented with vitamins. All the plants were grown under continuous white light at 22 °C. For the live imaging analysis of transgenic *pML1::GR-LhG4_p6xOp::REVr-2xVENUS* and *pUBQ10::GR-LhG4_ p6xOp::MIR165a* lines, seeds were sowed on GM plates supplemented with vitamins and 10 μM DEX or 0.1% ethanol (v/v) (mock).

Constructions for the transgenic plants

The *pMIR165a::mTagBFP-ER* transgene was generated using a 2073 bp region upstream of the *MIR165a* transcription start site, based on the previously reported transgene *p165a-T4* (5), and amplified and cloned into pDONR-P4P1R by BP cloning (Invitrogen). The *MIR165a* promoter was fused to the endoplasmic reticulum (ER)-localized mTagBFP, previously amplified and BP cloned into pDONR221, by LR recombination using pHGW as binary vector (Invitrogen). The *pMIR165a(-cis)::mTagBFP-ER* transgene was generated using the same cloning system as for *pMIR165a::mTagBFP-ER* and based on the previously reported transgene *p165a-T5* (5). The ER-localized GFP reporter for *MIR166a* (*pMIR166a::GFP-ER***)** was a gift from K. Nakajima (6).

To generate the miR165/166 fluorescent biosensors (*pUBQ10::REV-mEos2FP-ER* and *pUBQ10::REVi-mEos2FP-ER*) we used a previously reported design (7, 8). A 2 kb region of the 5' regulatory sequence from the *UBQ10* gene (*AT4G05230*) (9) was blunt-cloned upstream of the *REV-mEos2FP-ER* and *REVi-mEos2FP-ER* genes. Both the miR165/166 sensitive (*REV-mEos2FP-ER*) and miR165/166 insensitive (*REVi-*

mEos2FP-ER) biosensors were cloned into a sulfadiazine-resistant T-DNA vector by NotI site.

The *pREV::REV-2xYPET* transgene was generated by PCR amplification of a 7972 bp genomic fragment from the *REV* locus with primers 5'-GGATCCAGAGATAAATGGGTGTAAATGTGTG-3' and 5'-CCATGGACACAAAAGACCAGTTTACAAAGGA-3', and cloned as a translational fusion upstream of a 9 alanine linker followed by two tandem copies of YPET (10). This fusion protein appears to function to complement the *rev-1* mutant. The transgene was excised with NotI and cloned into the vector pDONR-P1P5r (Invitrogen) to generate the entry clone REV-2xYPET-L1R5. The *pPIN1::PIN1-GFP* transgene previously described (11) was excised with NotI and cloned into the vector pDONR-P5P2 (Invitrogen) to generate the entry clone PIN1-GFP-L5L2. Combination of REV-2xYPET-L1R5 and PIN1-GFP-L5L2 entry clones was assembled with the destination vector pBGW by LR recombination (Invitrogen).

For the construction of the *pHAT3::VENUS-HAT3* reporter, the *HAT3* genomic coding region (1583 bp downstream of the ATG) was PCR amplified using primers 5'-agatctAACAATGAGTGAAAGAGATGATGGATTGG-3' and 5'-actagtCTAATGAGAACCAGCAGCAGGTC-3', and cloned downstream of VENUS followed by a 9 alanine linker using BglII and SpeI restriction sites. A 5826 bp region of the upstream regulatory sequence from *HAT3* was PCR amplified using primers 5'-ggtaccCCCTAAGTAGCTAAAGAAAAGCAGAAT-3' and 5'-ggatccTTTTCTCAACCCCAGAAAGAAGA-3' and cloned upstream of *VENUS-HAT3* by KpnI and BamHI restriction sites. The *pHAT3::VENUS-HAT3* construct was cloned into pMOA34 by NotI site. The same strategy was followed for the construction of the *pATHB4::VENUS-ATHB4* reporter using primers 5'-

agatctATGGGGGAAAGAGATGATGGGTTGG-3' and 5'-actagtCTAGCGACCTGATTTTTGCTGGAGA-3' to PCR amplify a 1639 bp coding region downstream of the ATG and primers 5'-ggtaccTAAGTTTAACCCAGATACCACCAGT-3' and 5'-ggatccGTCCTCAACAGAAAGAACTTTTC-3' to amplify a 4738 bp of the upstream regulatory sequence. Functionality was confirmed by identifying several T2 families double *hat3 athb4* homozygous in which the wild type phenotype strictly cosegregated with the transgene (Fig. S3 *A-F*). HAT3 and ATHB4 translational reporters were transformed into *pMIR165a::GFP-ER*-expressing plants (a gift from P. Benfey) (8) by *Agrobacterium tumefaciens*-mediated floral dipping.

The *VENUS-HAT3* translational fusion was cloned into the p6xOp/GR-LhG4 two-component system (12) for dexamethasone-inducible misexpression of *VENUS-HAT3*. A *pML1::GR-LhG4* driver containing 3.4 kb of the L1-specific *ML1* gene (*AT4G21750*) (13) fused to the chimeric GR-LhG4 transcription factor and a *p6xOp::VENUS-HAT3* expression construct were cloned into the vectors pDONR-P1P5r and pDONR-P5P2 (Invitrogen), respectively. Combination of both entry clones was assembled with the destination vector pBGW by LR recombination (Invitrogen) (*pML1>>VENUS-HAT3*).

A *pUBQ10::GR-LhG4* driver containing 2 kb of the 5' regulatory sequence from the *UBQ10* gene (*AT4G05230*) (9) and a *p6xOp::MIR165a* expression construct (14) were combined in a sulfadiazine resistant T-DNA vector (*pUBQ>>MIR165a*) and transformed into *pMIR166a::GFP-ER*-expressing plants (a gift from K. Nakajima) (6), and into the transgenic *pMIR165a::GFP-ER_ pHAT3::VENUS-HAT3* and *pMIR165a::GFP-ER_ pATHB4::VENUS-ATHB4* lines (see above).

The *pREV::REV-2xVENUS* transgene was generated by cloning a 7972 bp genomic fragment from the *REV* locus as a translational fusion upstream of a 9 alanine linker followed by two tandem copies of VENUS (15). A microRNA resistant version of *REV* cDNA previously described (2) was modified to generate *REVr* coding sequence by cloning a 1148 bp BamHI-XcmI microRNA resistant partial cDNA fragment into the wild type *REV-2xVENUS* transgene. The *pREV::REVr-2xVENUS* was generated by PCR amplification of the *REV* promoter with primers 5'-gtcgacTGGGTGTAAATGTGTGGTCAGCACG-3' and 5'-ggatccATCTCCATTTTAGCTCGACCCTC-3' and by cloning a SalI-BamHI fragment upstream of the *REVr-2xVENUS* translational fusion. Entire NotI fragments were cloned into a modified vector conferring sulfadiazine resistance in plants.

The *REVr-2xVENUS* translational fusion was cloned into the p6xOp/GR-LhG4 two-component system (12) for dexamethasone-inducible misexpression of *REVr-2xVENUS*. The *pML1::GR-LhG4* driver (13) and the *p6xOp::REVr-2xVENUS* expression construct were cloned into a sulfadiazine-resistant T-DNA vector (*pML1>>REVr-2xVENUS*). For live imaging analyses, the *pML1>>REVr-2xVENUS* transgene was transformed into *hat3 athb4* plants containing the *pMIR165a::mTagBFP-ER* transgene or into wild-type plants containing the *pMIR165a(-cis)::mTagBFP-ER* and the *pMIR165a::GFP-ER* transgenes mentioned above. For the ChIP-qPCR analysis, the *pML1>>REVr-2xVENUS* transgene was transformed into wild type plants.

Confocal microscopy and image analysis

Live imaging analyses were performed on a Leica SP5 confocal microscope (Leica, Germany) using a water-dipping 25x objective. Seedlings were imaged under water

on GM plus vitamins medium plates, and with the root and the hypocotyl embedded in the GM medium. To allow a proper exposition of the shoot apex during live imaging of 3, 4 or 5 day-old seedlings, one cotyledon was carefully removed. Live imaging of GFP, mEos2FP, YPET or VENUS proteins was carried out using an Argon laser with a 488 nm line together with a 498-517 nm collection for GFP and mEos2FP, and a 514 nm line for YPET and VENUS together with a 527-540 nm collection. Live imaging of mTagBFP was performed using a 405 nm diode laser in conjunction with a 412-467 nm collection. Autofluorescence was collected at 610-700 nm. To image GFP/YPET and mTagBFP/VENUS combinations, we used the conditions mentioned above for each channel, and sequential scanning in line-scan mode with a NF 514 filter. Finally, the acquired z-stacks from the confocal microscope were analyzed using Imaris 7.7.1 (Bitplane).

Small RNA northern blot

Total RNAs were isolated from 2 week-old Col-0, *p35S::miR165a*, *hat1 hat2* and *hat3 athb4* plants. Plant samples were ground in liquid nitrogen and mixed with 1 ml of Trizol (Invitrogen). After centrifugation of Trizol-dissolved samples at 15,000 rpm for 15 min, the aqueous phase was extracted and RNAs were precipitated twice with isopropanol (100% and then 75%) and resuspended in 50% (w/v) formamide. Purified RNAs were separated on a 12.5–15% denaturing polyacrylamide gel (National Diagnostics) and subsequently blotted onto a nylon membrane (Amersham). Blots were incubated for 12 h with a 5'-end radiolabelled DNA probe against miR165/166 (Ambion). Blots were washed twice with SSC (2X)/SDS (0.1%) for 20 min each. Hybridization signals were detected with a Typhoon trio phosphorimager (GE Healthcare).

RT-qPCR

For the measurement of mature miR165/166 levels after *HAT3* ectopic induction, transgenic *pML1::GR-LhG4_p6xOp::VENUS-HAT3* plants were used. Seeds were sowed on GM plates and transferred to 10 μM DEX or 0.1% ethanol (control) plates 7 days after germination (DAG). Tissue was collected 3 days after transferring to DEX or ethanol plates and only shoot apexes containing very young leaves were isolated for RNA extraction. To quantify mature miR165/166 levels after *REVr* misexpression in the presence of cycloheximide (CHX), p35S::GR-REVr plants (16, 17) were used. 10 day-old seedlings were immersed in a 20 μM CHX solution during 30 minutes and then in a 20 μM CHX solution supplied with 10 μM DEX or 0.1% ethanol (control) during 3 h. Simultaneously, 10 day-old seedlings were immersed in a 10 μM DEX or 0.1% ethanol solution during 3h for gene expression comparisons in the presence or absence of CHX. Shoot apexes were collected after 3 h of treatment for RNA extraction.

RNA extraction from three biological replicates (DEX and ethanol; DEX + CHX and ethanol + CHX) was carried out following the Direct-zol RNA MiniPrep kit instructions (Zymo Research). To quantify mature miRNAs levels, 100 ng of total RNA including small RNAs were used for the stem-loop pulsed reverse transcription following a protocol previously described (18). This method allowed measuring the expression level of the mature miR165/166 as well as *ACTIN2* (*ACT2*; *AT3G18780*; reference gene), by using appropriate stem-loop RT primers (see Table) and oligo(dT) in the same RT reaction. For real-time qPCR, a mixture containing 10 μl of SYBR Green PCR master mix (Applied Biosystems), 2 μM of forward and reverse primers, and the cDNA was amplified in a StepOnePlus Real-Time PCR System (Applied

Biosystems U.S.A). Real-time qPCR started with incubation at 95°C for 10 min and was followed by 40 cycles of incubations at 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. To amplify the mature miRNA165/166, the primer pair of miR165-SL-F or miR166-SL-F and Universal-SL-R were used (see Table). To measure the expression level of other mRNAs, 1 μg of total RNA was used to synthesize cDNA using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. For real-time qPCR, a PCR mixture containing 10 μl of SYBR Green PCR master mix (Applied Biosystems), 10 μM of forward and reverse primers, and the ChIP DNA was amplified using the same conditions described above. *ATHB2* and *AT1G20823* were amplified using pairs of primers previously reported (16, 19). Additional sequences of the forward and reverse primers are listed below. Relative gene expression was calculated as previously described (20).

List of primers for RT-qPCR

Primer name	**Sequence**
miR165-SL-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGGGAT
miR166-SL-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGGGAA
miR165-SL-F	TCGCTTCGGACCAGGCTTC
miR166-SL-F	TCGCTTCGGACCAGGCTTCA
Universal-SL-R	GTGCAGGGTCCGAGGT
HAT3-F	ACTGTACTGCTGCACAAGTGT
HAT3-R	AGCCCCAATCCATCATCTCTTT
ATHB4-F	AATCCGAAGCAAAAGCTGGC
ATHB4-R	TTTCTGCAGCCGTCGATTCT
ZPR3-F	ACTCGAAGCTGTTTGTTGAAA
ZPR3-R	AGATTGTCCAGAAGCAGAGC
ACT2-F	GCCATCCAAGCTGTTCTCTC
ACT2-R	CAGTAAGGTCACGTCCAGCA

Yeast-two-hybrid assay

The yeast-two-hybrid screening was performed by Hybrigenics Services, S.A.S., Paris, France (http://www.hybrigenics-services.com). The REV full-length cDNA was cloned into pB27 as a C-terminal fusion to LexA. pB27 derives from the original pBTM116 (Vojtek and Hollenberg, 1995) plasmid. The construct was checked by sequencing the entire insert and used as a bait to screen a random-primed Universal Arabidopsis Normalized cDNA library (Clontech). 33.2 millions clones and 27.2 millions clones were screened using a mating approach with Y187 (Clontech library) and L40DGal4 (MATa) yeast strains as previously described (Fromont-Racine et al., 1997). 158 and then 155 colonies grown on +His plates were next selected on a -Trp, -Leu, -His medium supplemented with 10 mM 3-AT to handle bait autoactivation. The prey fragments of the positive clones were amplified by PCR and sequenced at their 5' and 3' junctions. The resulting sequences were used to identify the corresponding interacting proteins in the GenBank database (NCBI) using a fully automated procedure. A confidence score (PBS, for Predicted Biological Score) was attributed to each interaction as previously described (Formstecher et al., 2005). HAT3 was isolated in the Y2H screening as a potential REV-interacting protein. However, the HAT3 clone had a deletion corresponding to the first 88 amino acids. We therefore repeated the Y2H assay using the HAT3 full-length cDNA and the same Y2H system. The Y2H assay was performed using the Clontech's standard yeast transformation protocol, and transformants were selected on -Trp, -Leu plates. Five transformed colonies per prey/bait combination were analyzed for their growth on -Trp, -Leu, -His, +3-AT plates as well as on -Trp, -Leu, +His, +3-AT plates using dilution series (1:1, 1:5, 1:10, 1:50) (Fig. 3*F*).

FRET-FLIM

For the FRET-FLIM studies, *GFP-REVOLUTA*, *RFP-HAT3* and *RFP-ZPR3* (positive control) (17) were expressed under the control of the *35S* promoter in leaf epidermal cells of 4 week-old tobacco plants (*Nicotiana benthamiana*) infiltrated with *Agrobacterium tumefaciens* cultures. Microscopy and FLIM analysis were performed 2 to 3 days post infiltration. Image and Fluorescence lifetime data were acquired using a Leica TCS SP8 confocal scanning microscope platform, combined with PicoHarp 300 TCSPC Module. For lifetime measurements, GFP-molecules were excited with light pulses from a PDL808-SC 470 nm Diode laser at a frequency of 10 kHz. At least 1500 counts/pixel were obtained and analyzed with the PicoQuant Sympho Time Software (version 5.3.2.2). Fluorescence lifetimes were fitted with a mono-exponential decay function (21). Average lifetime data of at least 10 nuclei is shown for each combination in Fig. 3*G*.

ChIP-qPCR

For ChIP-qPCR, transgenic *pML1::GR-LhG4_p6xOp::VENUS-HAT3* and *pML1::GR-LhG4_p6xOp::REVr-2xVENUS* plants were treated with DEX for 4 hours as previously described (22). *pML1::GR-LhG4_p6xOp::VENUS-HAT3* and *pML1::GR-LhG4_p6xOp::REVr-2xVENUS* plants treated with 0.1% ethanol (v/v) (mock) were used as control. Tissue fixation and nuclei preparation were carried out as previously described (23). Cross-linked DNA/protein complexes in the nuclei were fragmented by sonication with a Diagenode Bioruptor Sonicator. This fragmented chromatin prep was treated with anti-GFP antibody (ab290, Abcam) overnight. The IP, the reverse cross-linking and the purification of ChIP DNA was performed following the Zymo ChIP-Spin kit manufacturer's protocol (Zymo Research). The real-time

qPCR mixture and conditions are described in the ChIP-qPCR section. To identify REV and HAT3 binding enrichment in the vicinity of *MIR165a* the percent input method was used. *ACT2* and ZPR3 were also tested as negative and positive controls, respectively (24, 25). *ACT2* was amplified using pairs of primers previously reported (24). Additional sequences of the forward and reverse primers are listed below.

List of primers for ChIP-qPCR

Distance from the transcription start site (kb)	Primer name	Sequence
-1.3	MIR165a-I-F	GGGCGACCAAGTATTTAAACC
	MIR165a-I-R	ACAAACGGAGAAGACGATGC
-0.7	MIR165a-II-F	CGAATGTATATGTGTATGCGTGG
	MIR165a-II-R	GAAACCACAGGCTGCCAAAA
-0.02	MIR165a-III-F	ATCTTCGTCTCCGCCACTCAT
	MIR165a-III-R	TGAAATAGCTTAACCCTCATGATAATCGA
+0.2	MIR165a-IV-F	TGCCTCTGATCACCATTTATTG
	MIR165a-IV-R	GCAAGAAAGATTCAAAGTCATCAC
-0.8	ZPR3-F	TCCTTCTCTCTCTCTCACTCTCT
	ZPR3-R	AGCAAGAAACAGAAACAGAGCA

Yeast-one-hybrid assay

Full-length cDNAs for *HAT3*, *ATHB4* and *ATHB2* were cloned into pDONR201, and then cloned into pDEST-GADT7 through LR recombination (Invitrogen) to be used as the prey proteins. Full-length cDNAs for *REV*, *PHB* and *PHV* were first cloned into pDONOR221 (Invitrogen) through Gateway BP reaction, and then cloned into pDEST22 (Invitrogen) through Gateway LR reaction to create GAL4AD-REV fusion protein. A DNA stretch containing four repeats of a 80 nt long DNA sequence located 17 bp upstream of the *MIR165a* transcription start site (Fig. S9*A*) was synthesized and cloned in tandem into pUC57 by the company GeneScript. This 80 nt long sequence contains the *cis*-acting element involved in the polarity of *MIR165/166* expression (5)

(Fig. S9*A*). Similarly, four repeats of the same sequence containing a mutated version of the element, previously referred as M1 (5) (Fig. S9*A*), were synthesized and cloned in tandem into pMA by the MR. GENE company. Both the wild type and M1 baits were individually cloned into pYi2267OHIS (26) through XhoI to drive the expression of the *HIS3* reporter gene. This plasmid also contains a unique StuI site within the *URA3* gene, allowing directed integration at the *URA3* chromosomal gene (18). Thus, resulting wild type and M1 bait constructs were linearized with StuI and integrated into the genome of the yeast strain W303-1A (26), which contains a mutation in the *URA3* gene, allowing selection of transformants on –Ura medium. The integration of the bait construct in the yeast genome was confirmed by PCR amplification (Fig. S9*B*) and sequence analysis using primers at the 5' and 3' ends of the bait insertion (5'-ATCTGAGTTCCGACAACAATGAG-3' and 5'-CTAAAGTTGCCTGGCCATCCAC-3'). 15 mM and 2 mM 3-aminotriazole (3-AT) amount was used to stop background HIS3 activity in wild type and M1 bait strains, respectively. Different combinations of prey constructs were co-transformed into bait strains using Clontech's standard yeast transformation protocol, and transformants were selected on -Ura, -Trp, -Leu plates. Three transformed colonies per prey/bait combination were analyzed for their growth on -Ura, -Trp, -Leu, -His, +3-AT plates (Fig. 5*G* and S8*A*) as well as on -Ura, -Trp, -Leu, +His, +3-AT plates (Fig. S8*B*) using dilution series (1:5, 1:10, 1:20, 1:50).

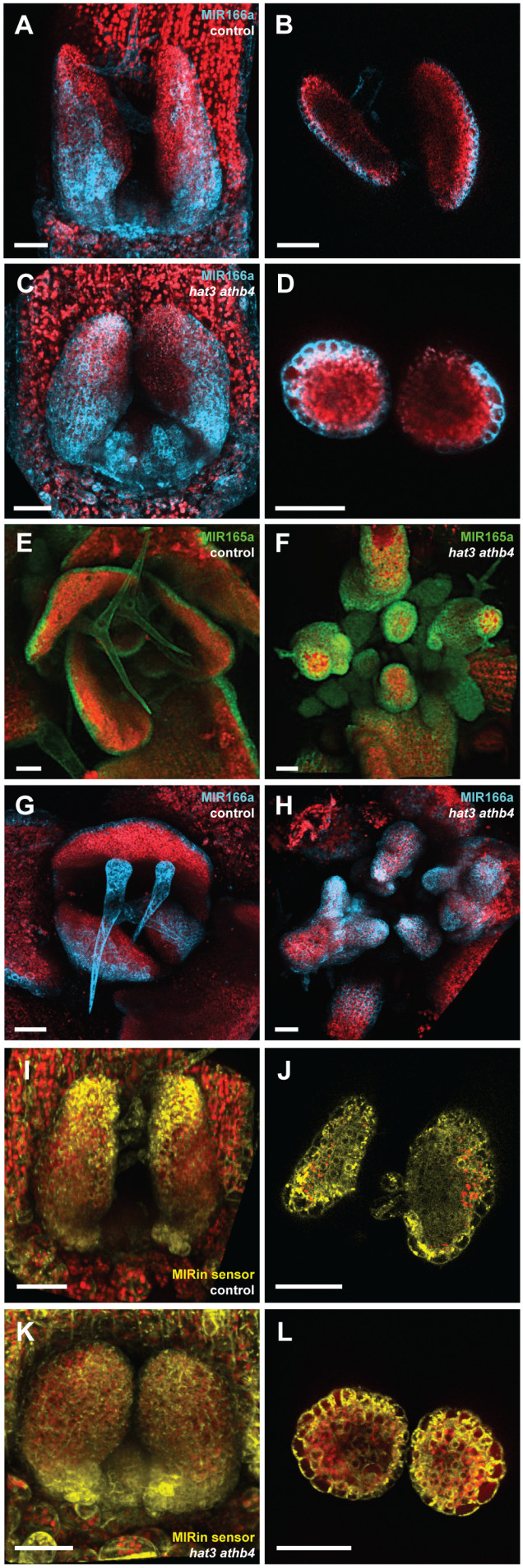
A
MIR166a
control
B
C
MIR166a
hat3 athb4
D
E
MIR165a
control
F
MIR165a
hat3 athb4
G
MIR166a
control
H
MIR166a
hat3 athb4
I
MIRin sensor
control
J
K
MIRin sensor
hat3 athb4
L

Fig. S1. HAT3 and ATHB4 regulate *MIR165a* and *MIR166a* expression. (*A*-*D*) Expression of *pMIR166a::GFP-ER* reporter (blue) in the shoot apex of 6 day-old control (*A*, *B*) and *hat3 athb4* plants (*C*-*D*). (*B*, *D*) Cross sections of the same leaf primordia shown in *A* and *C*, respectively. (*E*, *F*) Expression of *pMIR165a::BFP-ER* reporter (green) in the shoot apex of 15 day-old control (*E*) and *hat3 athb4* plants (*F*). (*G*-*H*) Expression of *pMIR166a::GFP-ER* reporter (blue) in the shoot apex of 15 day-old control (*G*) and *hat3 athb4* plants (*H*). (*I*-*L*) Expression of a miR165/166 insensitive biosensor (control; yellow) fused to mEos2FP (*pUBQ10::REVi-mEos2FP-ER*) in the shoot apex of 4 day-old control (*I*, *J*) and *hat3 athb4* plants (*K*, *L*). (*J*, *L*) Cross sections of the same leaf primordia shown in *I* and *K*, respectively. Chlorophyll autofluorescence: red. Scale bars: 50 μm.

Fig. S2. *rev-10d* gain-of-function mutation complements *hat3 athb4* mutant. (*A*) Phenotype of Col-0 plants. (*B*) *rev-10d* gain-of-function mutant develops flat leaves as wild type plants, however, displays alterations in the polar pattern of the vasculature (2). (*C*) *hat3 athb4* loss-of-function mutant shows radialized leaves. (*D*) *rev-10d* mutation attenuates the leaf phenotype of *hat3 athb4* mutant.

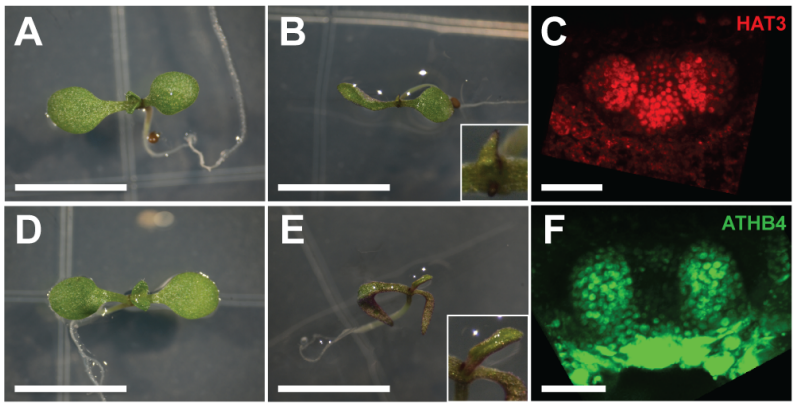

Fig. S3. Complementation of *hat3 athb4* mutant by HAT3 and ATHB4 translational reporters. (*A-F*) T2 generation of double homozygous *hat3 athb4* plants transformed with *pHAT3::VENUS-HAT3* (*A-C*) or *pATHB4::VENUS-ATHB4* reporters (*D-F*). (*A*, *D*) Hygromycin-resistant transformants show wild type-like phenotype. The cotyledon and leaf phenotype of *hat3 athb4* was completely rescued by *pHAT3::VENUS-HAT3* (*A*) and *pATHB4::VENUS-ATHB4* reporters (*D*). (*B*, *E*) *hat3 athb4* seedlings grown on GM medium show narrow cotyledons and radialized leaves (the first pair of leaves is shown in the magnified images; B and E). (*C*, *F*) Shoot apexes of transformant *hat3* athb4 seedlings show expression of *pHAT3::VENUS-HAT3* (*C*; red) and *pATHB4::VENUS-ATHB4* reporters (*F*; green). Scale bars: 5 mm (*A*, *B*, *D*, *E*) and 50 μm (*C*, *F*).

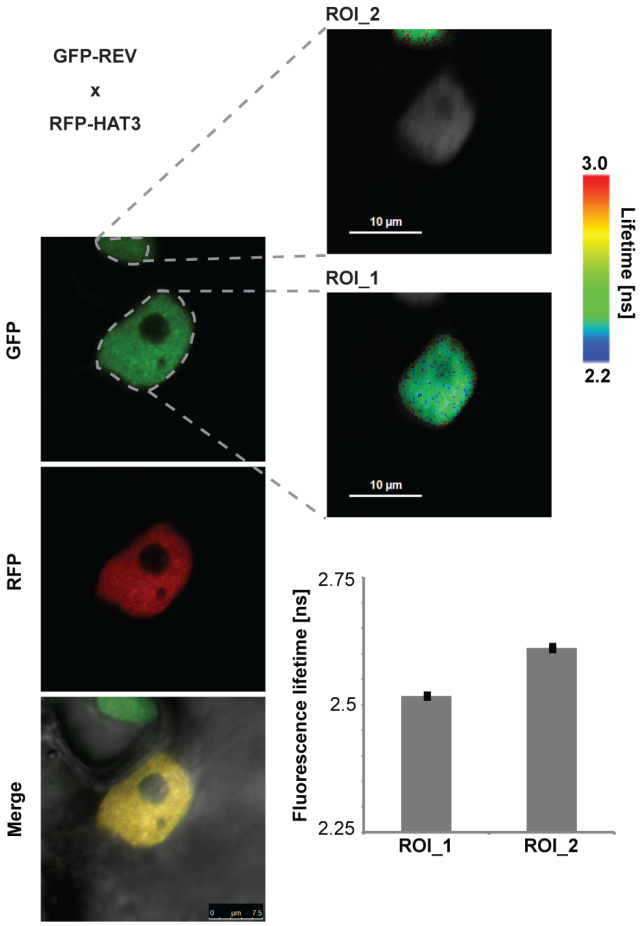

Fig. S4. Additional GFP fluorescence lifetime quantification in a FRET-FLIM assay. GFP fluorescence lifetime (ns) quantification of a nucleus co-expressing GFP-REV and RFP-HAT3 (Region Of Interest 1; ROI_1) and a nucleus with no detectable RFP signal (ROI_2) is shown. Error bars show mean of FLIM measurements within ROI_1 or ROI_2 ± standard deviation. Scale bars: 7.5 and 10 µm.

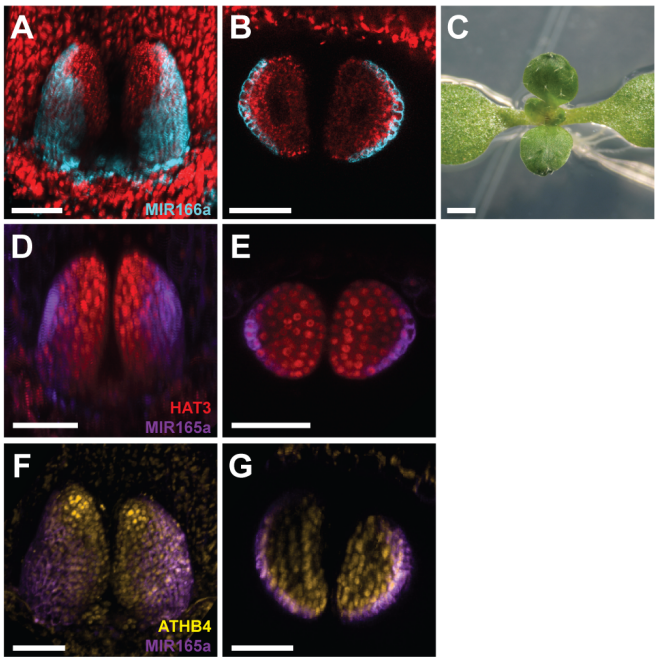

Fig. S5. Expression pattern of *MIR166a*, *MIR165a*, *HAT3* and *ATHB4* reporters in plants transformed with *pUBQ10::GR*-LhG4 and *p6xOp::MIR165a* constructs grown on ethanol-GM medium (control). (*A*) Expression of *pMIR166a::GFP-ER* reporter (blue) in the shoot apex of Col-0 plants 4 days after germination on GM plates containing 0.1% ethanol (mock). *MIR165a* expression was not induced in these control conditions. (*B*) Cross section of the same leaf primordia shown in *A*. (*C*) Phenotype of plants 7 days after germination on control medium. (*D*) Expression of *pMIR165a::GFP-ER* (purple) and *pHAT3::VENUS-HAT3* (red) reporters in the shoot apex of Col-0 plants 4 days after germination on GM plates containing 0.1% ethanol. (*F*) Expression of *pMIR165a::GFP-ER* (purple) and *pATHB4::VENUS-ATHB4* (yellow) reporters in the shoot apex of Col-0 plants 4 days after germination on GM plates containing 0.1% ethanol. (*E*, *G*) Cross sections of the same leaf primordia shown in *D* and *F*. Chlorophyll autofluorescence: red (*A*, *B*). Scale bars: 50 μm (*A*, *B*, *D*, *E*, *F*, *G*); 5 mm (*C*).

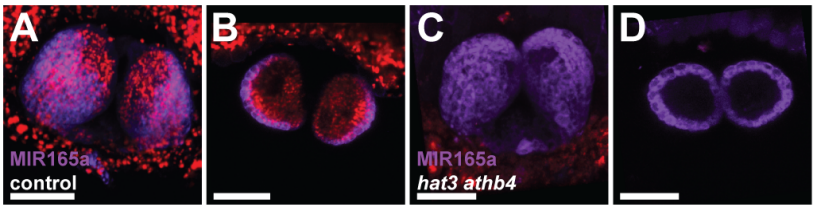

Fig. S6. Expression pattern of *MIR165a* transcriptional reporter in control and *hat3 athb4* plants containing *pML1::GR-LhG4* and *p6xOp::REVr-2xVENUS* constructs grown on ethanol-GM medium. (*A*, *D*) Expression of *pMIR165a::BFP-ER* reporter (purple) in the shoot apex of control (*A*, *B*) and *hat3 athb4* plants (*C*, *D*) 4 days after germination on GM plates containing 0.1% ethanol (mock). *REVr-2xVENUS* expression was not induced in these control conditions. (*B*, *D*) Cross sections of the same leaf primordia shown in *A* and *C*, respectively. Chlorophyll autofluorescence: red. Scale bars: 50 μm.

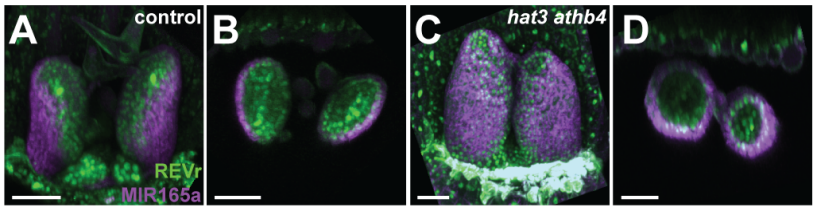

Fig. S7. Expression pattern of *MIR165a* transcriptional reporter in control and *hat3 athb4* plants transformed with *pREV::REVr-2xVENUS*. (*A*, *D*) Expression of *pMIR165a::BFP-ER* reporter (purple) and *pREV::REVr-2xVENUS* (green) in the shoot apex of control (*A*, *B*) and *hat3 athb4* plants (*C*, *D*) 4 days after germination. (*B*, *D*) Cross sections of the same leaf primordia shown in *A* and *B*, respectively. Scale bars: 50 μm.

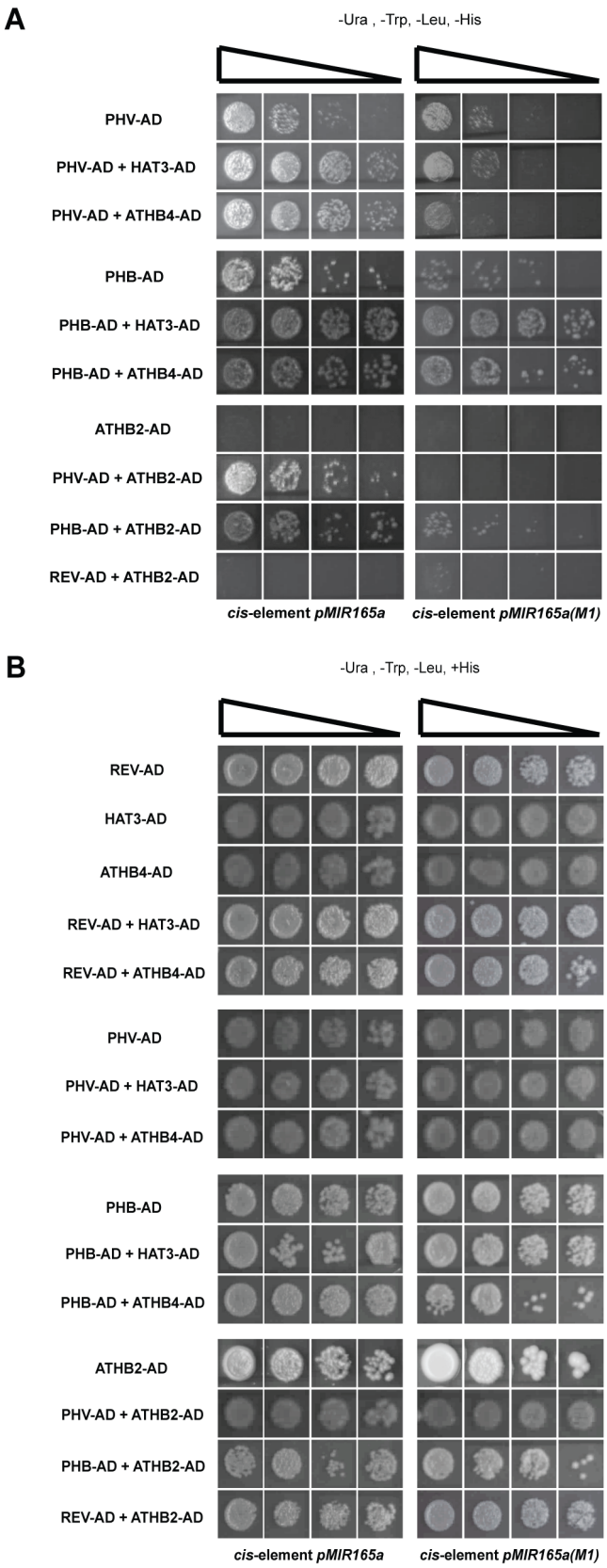
A
-Ura , -Trp, -Leu, -His
PHV-AD
PHV-AD + HAT3-AD
PHV-AD + ATHB4-AD
PHB-AD
PHB-AD + HAT3-AD
PHB-AD + ATHB4-AD
ATHB2-AD
PHV-AD + ATHB2-AD
PHB-AD + ATHB2-AD
REV-AD + ATHB2-AD
cis-element pMIR165a
cis-element pMIR165a(M1)
B
-Ura , -Trp, -Leu, +His
REV-AD
HAT3-AD
ATHB4-AD
REV-AD + HAT3-AD
REV-AD + ATHB4-AD
PHV-AD
PHV-AD + HAT3-AD
PHV-AD + ATHB4-AD
PHB-AD
PHB-AD + HAT3-AD
PHB-AD + ATHB4-AD
ATHB2-AD
PHV-AD + ATHB2-AD
PHB-AD + ATHB2-AD
REV-AD + ATHB2-AD
cis-element pMIR165a
cis-element pMIR165a(M1)

Fig. S8. Additional interactions of HD-ZIPIIs and HD-ZIPIIIs using yeast-one-hybrid assays and controls. (*A*) Interaction of additional HD-ZIPIIs and HD-ZIPIIIs with the *cis*-element in the *MIR165a* promoter in a yeast-one-hybrid assay. Three transformed colonies per prey/bait combination were analyzed for their growth on -Ura, -Trp, -Leu, -His, +3-AT selection plates using dilution series (1:5, 1:10, 1:20, 1:50). (*B*) Growth of yeast on non-selective medium. An aliquot of yeast culture used in Fig. 5*G* and Fig. S8*A* was simultaneously grown on -Ura, -Trp, -Leu, + His, +3-AT plates as a control. AD, activation domain.

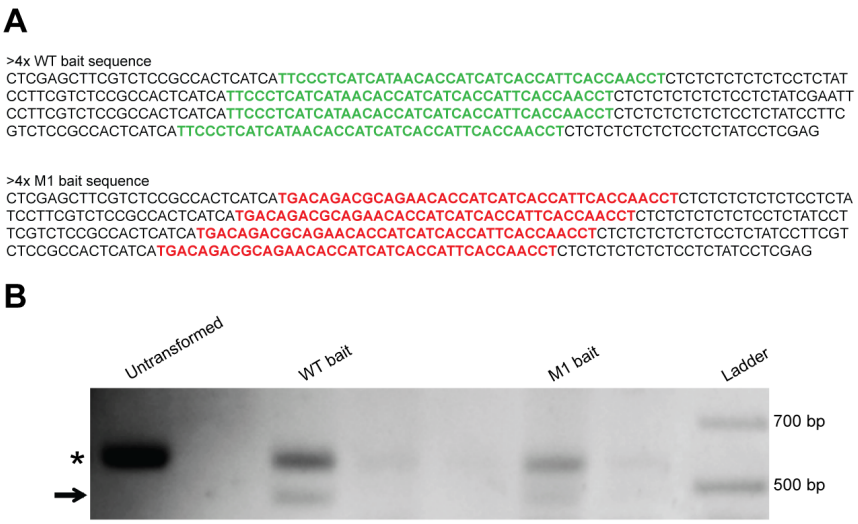

Fig. S9. Yeast-one-hybrid bait constructs. (*A*) DNA stretch containing four repeats of a 80 nt long genomic sequence located 17 bp upstream of the *MIR165a* transcription start site. This 80 nt long sequence contains the *cis*-acting element (green) involved in the polarity of *MIR165/166* expression (5). Four repeats of the same sequence containing a mutated version of the element (red; negative control), previously referred as M1 (5), is also shown. (*B*) Confirmation of the bait construct integration in the yeast genome by PCR amplification. Arrow, wild type and M1 bait insertions; asterisk, unspecific PCR product.

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