Supplementary Information

Arabidopsis BTB/POZ protein-dependent PENETRATION3 trafficking and disease susceptibility

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Supplementary Methods

Plant materials and growth conditions. We used wild-type plants of the *Arabidopsis thaliana* accessions Columbia-0 (Col-0) and Landsberg *erecta* (L*er*). A transgenic line expressing H2B-mCherry in the background of *pPEN3:PEN3-GFP; pen3-1*[1] was used to perform the genetic screen[9]. The T-DNA insertion line *eap3-2* was obtained from the Nottingham Arabidopsis Stock Centre (NASC). The primers used for genotyping this line were 1326SacIF1 and 1326R1, and Lba1 (Supplementary Information Table 2). Other marker lines were obtained from the corresponding authors of cited publications: *pPEN3:PEN3-GFP pen3-1*[1], *p35S:EGFP-LTI6a*[21], *pPIN2:PIN2-EGFP*[22], *pPEN3:PEN3-mCherry*[9], *pUBQ10:NIP5;1-mCherry*[23], *p35S:PIS1-GFP*[24], *p35S:ER-ECFP-HDEL*[25]. Plant growth conditions were as described previously[26] with minor modifications. In brief, seeds were surface sterilized and stratified at 4°C for 2 or 3 days prior to plating on ½x MS plates (½x Murashige and Skoog, 1% Sucrose, 2.5 mM morpholinoethanesulfonic acid (Sigma-Aldrich, St. Louis, MO, USA), 0.8% plant agar, pH 5.8). Seedlings were grown vertically at 23°C day and 18°C night under 16 h light / 8 h dark cycle for 5 days before analysis.

Mapping and identification of the *eap3-1* mutation. Seeds expressing the pPEN3:PEN3-GFP and the p35S:H2B-mCherry transgenes were mutagenized as

described[9]. Five-day-old seedlings of each M2 line were examined with a Leica TCS SP2 AOBS spectral confocal laser scanning system (Leica Microsystems, Wetzlar, Germany). *eap3-1* was isolated as a line displaying mislocalization of PEN3-GFP. The *eap3-1* mutant was crossed with L*er* to generate an F2 mapping population from which 260 out of 1054 (24.6%) seedlings displayed the PEN3-GFP ER-mislocalization phenotype identified by CLSM observation, indicating it was caused by a single recessive mutation. SSLP (simple sequence length polymorphism) and dCAPS (derived cleaved amplified polymorphic sequence) molecular markers were generated as described[27] and by using The Arabidopsis Information Resource (http://www.arabidopsis.org), and the mutation was mapped to a 275 kb interval on chromosome 3.

For mapping-by-sequencing mapping, 259 plants from an outcrossing F2 mapping population were pooled and thoroughly ground in liquid nitrogen. 24 plants of the non-mutagenized parental line were also pooled and ground in liquid nitrogen. 10% of total ground tissue were subjected to genomic DNA (gDNA) extraction employing the column-based DNeasy kit (Qiagen, Hilden, Germany). After fluorometric quantification with Qubit 2.0 (Thermo Fisher Scientific, Waltham, MA, USA), 100 ng of gDNA were employed for library preparation, following the protocol of the TruSeq Nano kit (Illumina, San Diego, CA, USA).

The sample was sequenced barcoded on half a lane (2-plexed) on an Illumina HiSeq 2000 (Illumina, San Diego, CA, USA). Demultiplexing was performed using Illumina's bcl2fastq script. The software pipeline SHORE v.0.9.0 with its default options was used to trim and filter the reads by quality, low complexity, and number of N’s. These steps resulted in 188,195,068 reads. The reads were aligned against the Col-0 genome (TAIR9) allowing for 10% mismatches or gaps relative to the read

length using SHORE and GenomeMapper v0.4.5s, resulting in a whole-genome average coverage of 130x. We found 309 high quality (Q25) homozygous, putative EMS-induced SNPs (C-T or G-A mismatches). We performed a SHOREmap analysis[28] plotting the allele frequency of the Col-0 base (in 200 kbp sliding windows) at 461,070 marker positions. The markers are variable positions between Col-0 and L*er* and covered by at least one read in the L*er* sample. The average allele frequency along the chromosomes was almost exclusively 0.5 with the exception of one peak on chromosome 3. Inspecting the homozygous high quality SNPs in a 0.5-Mb interval around the peak resulted in two putative EMS-induced SNPs, of which one was a putative synonymous EMS-induced SNP located ~150,000 bases apart from the peak in the 3'UTR of gene AT3G09440. The other homozygous SNP in the gene AT3G09030 was predicted to be non-synonymous, located less than 3,000 base pairs from the peak position. This variant was selected as the obvious candidate for *EAP3*.

Immunofluorescence labelling and CLSM analyses. Whole-mount immunolabelling of roots was performed and analysed by immunofluorescence microscopy as described[26]. Antibody dilutions were rabbit anti-BIP 1:600 (Agrisera, Vännäs, Sweden); DyLight633 donkey anti-rabbit 1:100 (AS122034, Agrisera). Imaging of immunolabelled or live roots was carried out using a Zeiss LSM 780 confocal laser scanning system mounted on a Zeiss Axio Observer Z1 inverted microscope, employing a water-corrected C-Apochromat 40 × objective, numerical aperture 1.2 (Zeiss). Laser excitation lines for the different fluorophores were 405 nm for CFP, 488 nm for GFP, 561 nm for mCherry, and 633 nm for DyLight633. Fluorescence emission was detected at 437-483 nm for CFP, 491-552 nm for GFP,

587-631 nm for mCherry, and 636-699 nm for DyLight633. Detection was in sequential line-scanning mode with a line average of 4. Images were processed and analysed with Fiji software (http://www.imagej.nih.gov/ij/) or Adobe Photoshop CS5 (Adobe Systems, Mountain View, CA, USA), and assembled in Adobe Illustrator CS5 (Adobe Systems) using the freehand selection function in Fiji software (http://www.imagej.nih.gov/ij/).

Quantification of PEN3-mCherry localization. In order to quantitatively analyse the effect of EAP3-myc and EAP3-GFP on PEN3-mCherry localization, PEN3-mCherry intensities at the outer lateral plasma membrane domain were measured using segmented line function in Fiji software (http://www.imagej.nih.gov/ij/) along outer lateral plasma membrane. For fluorescence intensities in the cytoplasmic area, freehand selection function in Fiji was used to select the whole cytoplasmic area surrounded by the plasma membrane. The ratio between the mean intensities of outer lateral plasma membrane domain and cytoplasmic region was then calculated. On average two cells per root were selected per measurement making up to a final number of $n = 30$ cells from 15 roots. Student's two-tailed *t*-test with equal variance was employed to assess significances of differences.

Immunoprecipitation and Western blot analyses. Proteins were extracted using extraction buffer containing 25 mM Tris (pH 7.8), 75 mM NaCl, 10 mM $MgCl_2$, 5 mM EGTA, 0.1 mM Na_3VO_4, 10% glycerol, 0.2% NP40, 60 mM beta-glycerophosphate, 2 mM DTT, and the protease inhibitor cocktail. Protein quantification was performed using Bradford reagent (Bio-Rad Laboratories Inc, Hercules, CA, USA). GFP-Trap_MA beads (Cat No. gtma10; ChromoTeK,

Martinsried, Germany) or Myc-Trap beads (Cat No. yta10, ChromoTeK) were equilibrated with dilution buffer containing 10 mM Tris (pH 7.5), 150 mM NaCl, and 0.5 mM EDTA. Then the beads were added to the protein samples for 1 h at 4°C. After beads were washed three times with the wash buffer containing 10 mM Tris (pH 7.5), 300 mM NaCl, and 0.5 mM EDTA, proteins were eluted using 200 mM glycine (pH 2.5) and subjected to Western blotting. For Western blotting, the protein samples were heated at 70 °C for 30 min and loaded on a 4-15% Mini-PROTEAN TGX precast polyacrylamide gels (Bio-Rad). After electrophoresis at 80 V for 2 h, proteins were transferred to a 0.45 µm PVDF membrane (Immubilon-P, Sigma-Aldrich). The primary antibodies and dilutions used were: mouse monoclonal anti-Myc,1:1000 (9E10; Santa Cruz Biotechnology, Dallas, TX, USA), mouse anti-GFP, 1:5000 (Cat No. MMS-118R; Covance, Princeton, NJ, USA), rabbit anti-RFP, 1:1000 (Code No. PM005; Medical & Biological Laboratories, Nagoya, Japan), and rabbit anti-ACT, 1:2500 (AS132640; Agrisera). The secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse IgG, 1:10000 (Agrisera), and horseradish peroxidase-conjugated goat anti-rabbit IgG, 1:10000 (Agrisera). An ECL Western blotting detection reagent kit (Cat No. 34079; ThermoFisher Scientific) was used for chemiluminescent detection. Images were taken on Fujifilm LAS-3000 Imager (Fujifilm Life Science, Tokyo, Japan) and analysed with Fiji software (http://www.imagej.nih.gov/ij/) or Adobe Photoshop CS5 (Adobe Systems), and assembled in Adobe Illustrator CS5 (Adobe Systems).

Quantification of PEN3-mCherry protein levels. Protein quantification was performed in Fiji software (http://www.imagej.nih.gov/ij/). Briefly, Western blot images were converted from TIFF to JPEG file format in greyscale mode in Adobe

Photoshop CS5 (Adobe Systems). Then JPEG files were opened in Fiji software for measurement. To do this, "mean grey value" was chosen from the analyse menu "set measurement" in Fiji. Then the rectangular tool was selected to draw a frame covering the band for each lane and measurements were performed by clicking "measure" under the "analyse" menu in Fiji. The same frame was moved to the next lanes and measurement continued over the blot. After measurements of proteins of interest and loading controls, the pixel intensities were exported into an Excel table for analysis. Prior to calculation, the pixel intensities for all data were converted by 255 – X, where the X value was recorded by Fiji and 255 refers to the white background. A net protein of interest band value was calculated as (255 – Xp) – (255 - Xb), where Xp refers to the protein of interest value and Xb to the blot background recorded by Fiji. The same procedure was performed for loading controls expressed as (255 – Xc) – (255 - Xb). The final relative protein level was calculated as {(255 – Xp) – (255 - Xb)} / {(255 – Xc) – (255 - Xb)}.

RT-PCR analysis. RT-PCR analysis was performed as described, previously[26], with minor modifications. Cauline leaves were collected from 5-week-old plants. DNase treatment (DNA-free™, Ambion, TX, USA) was performed on 1 µg total RNA. cDNA synthesis was performed using the iScriPt™ cDNA Synthesis Kit (Bio-Rad,). Primers for *EAP3* were EAP3RTF1 and EAP3RTR1, for *ACT7*, ACT7F1 and ACT7R1 (Supplementary Table 2).

Developmental map of transcript levels employing the eFP Broser tool. *EAP3, PEN3* and *ACT7* transcript levels were investigated using the Arabidopsis eFP Browser tool at http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi. Transcript signal values

for *EAP3*, *ACT7* and *PEN3* from samples of different tissues at different developmental stages were extracted from databases[32,33] employing the AGI ID Arabidopsis Genome Initiative (AGI) identifier (ID) At3g09030, At5g09810, and At1g59870. These were individually used as the "Primary Gene ID" chosing "Absolute" mode and "Developmental Map" as data source clicking "Go" and subsequently "Click Here for Table of Expression Values" to extract average signal values and SDs for different tissues/developmental stages to chart them in Microsoft Excel for display in Supplementary Fig. 3.

Plasmid construction and transformation. To generate an EAP3 fusion to GFP, three PCRs were performed to obtain three fragments with overlapping enzyme sites. Primer pair 1326ECORIF1 and 1326ApaIR1 were employed to amplify fragment A1 including 1131 bp upstream and 1377 bp downstream of the ATG of the *EAP3* gene. Fragment A2 encompassing 1319 bp downstream of the *EAP3* stop codon was amplified with the primer pair 1326SalIF1 and 1326KpnIR1. The third fragment A3 containing the coding sequence of GFP was amplified with primer pairs ApaIGFPF and SalIGFPR. In this way, 15 amino acids serving as a linker between EAP3 and GFP were added prior to the ATG of GFP. The resulting fragments were separately subcloned into pBluescript SKII and the resulting plasmids sequenced by MWG (MWG, Biotech AG, Ebersberg, Germany). Finally, A1 was digested by EcoRI and ApaI, A2 was digested by SalI and KpnI, and A3 was digested by ApaI and SalI. These three fragments were concomitantly ligated into the EcoRI and KpnI sites of pGREENII0229 (John Innes Centre, Norwich, UK)[34]. To generate the coding sequence of EAP3 tagged with a myc epitope[35], a fragment of EAP3 called B2 including 1319 bp downstream of the stop codon was amplified with primer pair

CMycApaIF and 1326KpnIR1 and subcloned into pBluescript SKII. Fragment A1 in pBluescript SKII was digested by EcoRI and ApaI, and B2 was digested by ApaI and KpnI. These two fragments were ligated into pGREENII0229 digested by EcoRI and KpnI. All primer sequences are displayed in Supplementary Table 2. After sequencing, the final constructs were introduced into *Agrobacterium tumefaciens* strain GV3101, which was used for transformation into the *eap3-2 pPEN3:PEN3-mCherry* background by floral dipping[36].

***Fusarium oxysporum* root infection assays in soil**. For infection assays, *Fusarium oxysporum f. sp. conglutinans*, strain 699 (ATCC 58110)[37] was grown on potato dextrose agar (PDA) at 28°C. After 4-5 days, mycelium was inoculated onto liquid sucrose sodium nitrate medium[38] (containing per 1 liter, 15 g sucrose, 2 g $NaNO_3$, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl and the following trace elements 249 µg $FeSO_4.7H_2O$, 40 µg $CuSO_4.5H_2O$, 44 µg $ZnSO_4.7H_2O$, 31 µg $MnSO_4.H_2O$, 51 µg $NaMoO_4.2H_2O$, shaken at 150 rpm, 28°C for 3-5 days to produce conidia. Subsequently, conidia were separated by a double layer of miracloth (Millipore, Billerica, MA, USA). The flow-through was centrifuged at 1,700 g, the pellet was resuspended in dH_2O at a concentration of 10^7 conidia per ml. 3-week-old *Arabidopsis thaliana* plants grown on soil composed of 5 volumes peat and 1 volume perlite were inoculated with *Fusarium oxysporum f. sp. conglutinans*. Plants were infected by pipetting 10 ml of 10^7 conidia ml^{-1} suspension directly into the soil contained in a 125 ml plastic pot harbouring a single plant. Fresh weight and disease sensitivity score was obtained by measuring rosette weight and by observing chlorotic and necrotic symptoms scored at a scale from 1-10 as number of affected leaves per plant, respectively, two weeks after inoculation. 15-30 plants were employed per

genotype per experiments in each of three independent experiments (n = 3) from which averages and SDs were obtained. Statistical analyses were performed employing one-tailed Student's *t*-test with equal variance with the null hypothesis being that the mutants and transgenic lines would not show increased disease susceptibility or loss of fresh weight relative to wild type. The significance level indicating a significant difference between genotypes was $P < 0.05$ (n = 3).

Supplementary Methods References

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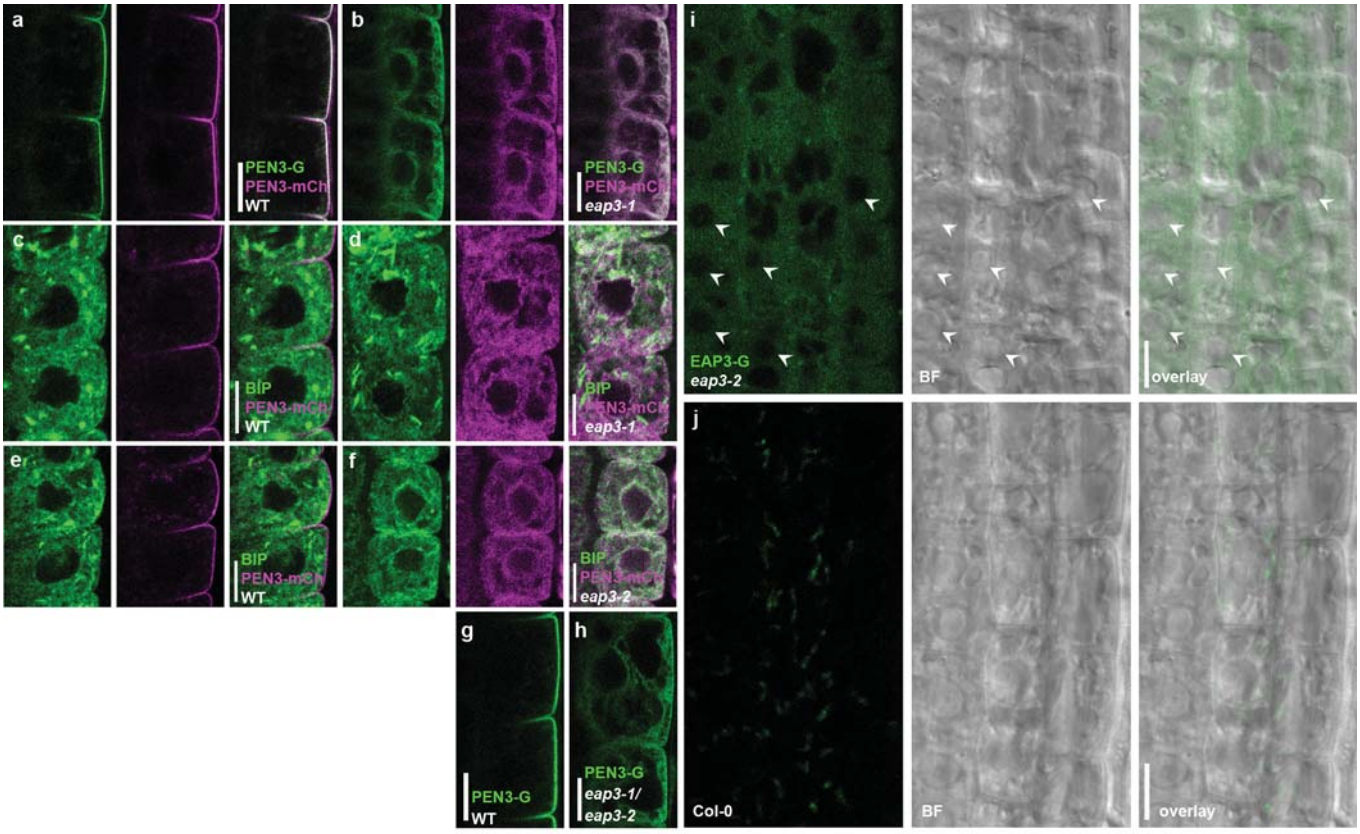
Supplementary Table 1| Significances of differences in disease sensitivity between different backgrounds.

Comparison	*P* values for Disease resistance	*P* values for Fresh weight
Col-0 vs *pen3-4*	0.005	0.002
Col-0 vs *eap3-2*	0.002	0.004
Col-0 vs *PEN3-mCherry eap3-2*	0.000	0.142
Col-0 vs *EAP3-myc PEN3-mCherry eap3-2*	0.478	0.270
Col-0 vs *PEN3-mCherry* Col-0	0.285	0.409
eap3-2 vs *PEN3-mCherry eap3-2*	0.101	0.159
eap3-2 vs *EAP3-mycPEN3-mCherry eap3-2*	0.001	0.042
PEN3-mCherry eap3-2 vs *EAP3-mycPEN3-mCherry eap3-2*	0.000	0.131
EAP3-mycPEN3-mCherry eap3-2 vs *PEN3-mCherry* Col-0	0.284	0.420
PEN3-mCherry eap3-2 vs *PEN3-mCherry* Col-0	0.033	0.232

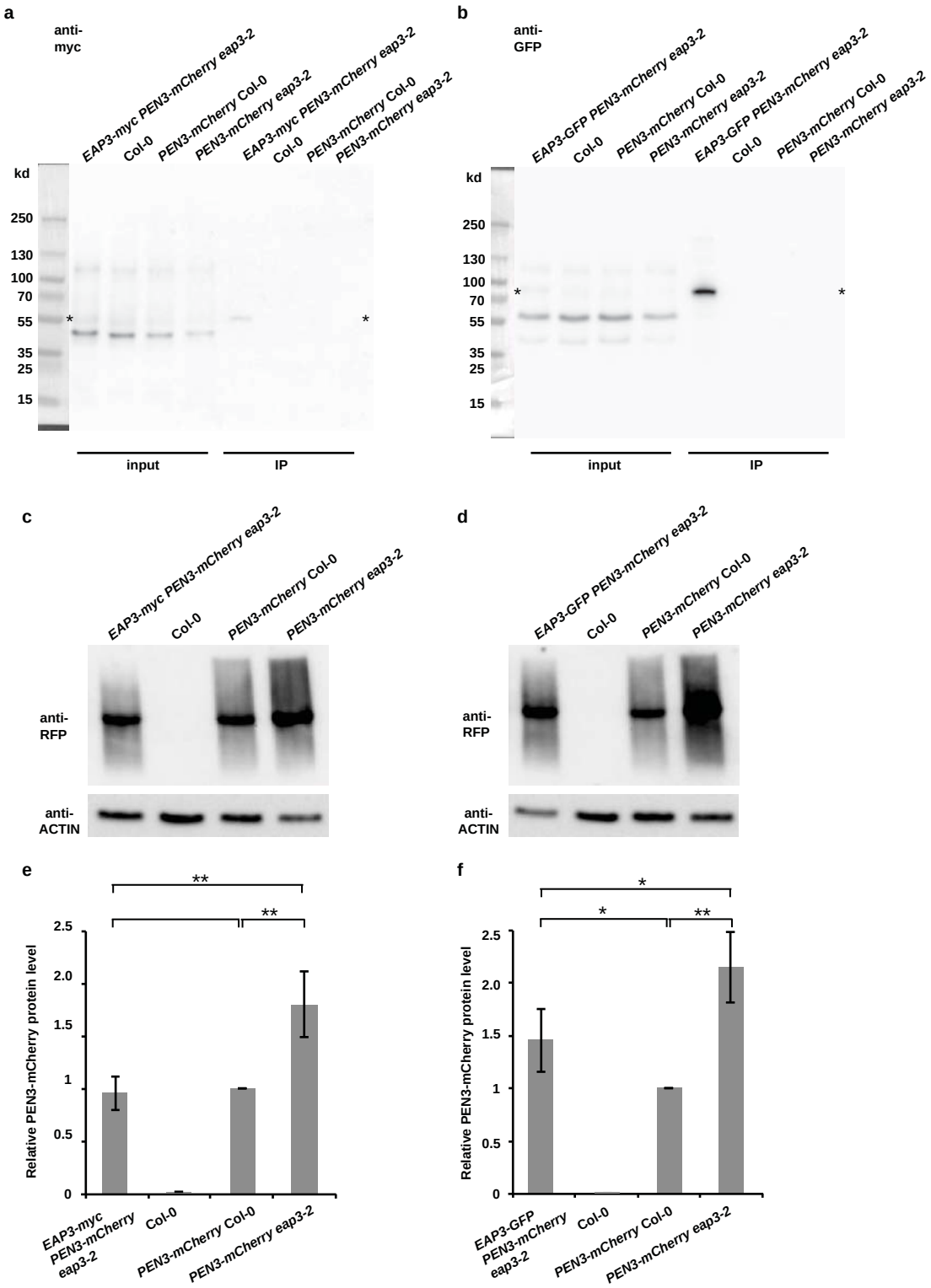
P values were determined by Student's one-tailed *t*-test with equal variance.

Supplementary Table 2 | Primers used in this study.

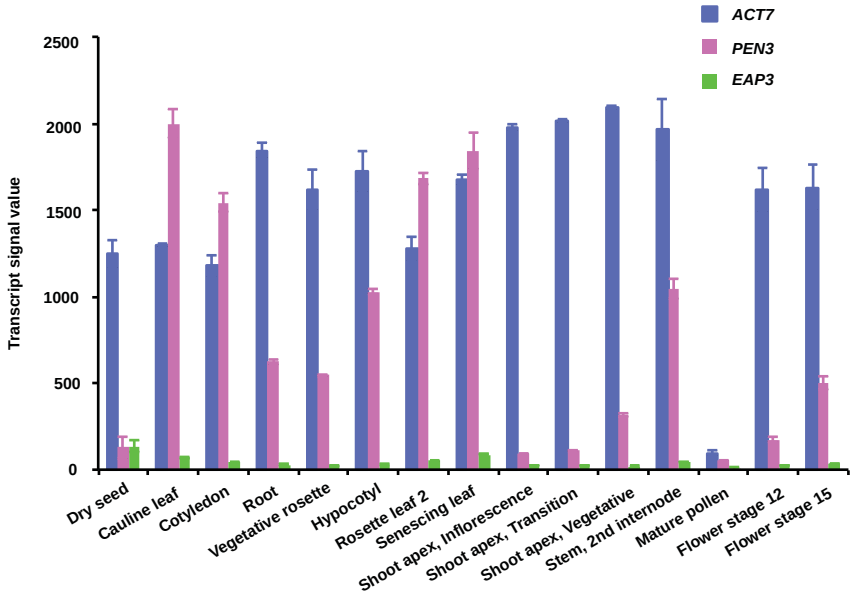
Primer name	Sequence	Used for
NGA6F	ATG GAG AAG CTT ACA CTG ATC	mapping, SSLP marker
NGA6R	TGG ATT TCT TCC TCT CTT CAC	
NGA172F	CAT CCG AAT GCC ATT GTT C	mapping, SSLP marker
NGA172R	AGC TGC TTC CTT ATA GCG TCC	
NGA162F	CTC TGT CAC TCT TTT CCT CTG G	mapping, SSLP marker
NGA162R	CAT GCA ATT TGC ATC TGA GG	
dM1F	AATGCAGAGAGATAAACCTCATCCaT	mapping, dCAPS marker
dM1R	TGAGACCGGGAGGGTTACTGTGGA	
dM2F	AGGCGTTCCGGGTGATAAGAATGCAGAtC	mapping, dCAPS marker
dM2R	TTGAGCACctagagagagatcattactga	
dM3F	TTCATATCCGGTCACGTCTTCGCT	mapping, dCAPS marker
dM3R	agagaagtgagaaagaatggagattggttctag	
dM4F	AGCATCGAAACCTATTTTCCAGAGTaCTAG	mapping, dCAPS marker
dM4R	TGGACTCAACGAGACTATAGCTCTGA	
dM5F	CAATGCTGGAAATAGGCCATCTGGTGA	mapping, dCAPS marker
dM5R	ctgaaatgatgatgtgtgaggctatctgga	
dM6F	GGATGCGAAATAAGCGATGA	mapping, dCAPS marker
dM6R	GGTGTAGCCGGCGTAAGTAA	
Lba1	GGGTGATGGTTCACGTAGTGGGCCATCG	genotyping of a T-DNA line
1326SacIF1	TACGATTGGAGTCTTTCCCATGCTGGA	genotyping of a T-DNA line
1326R1	AGTCTTCTACTAGACCCTGGA	genotyping of a T-DNA line
1326EcoRIF1	gaattcGGATCTCACCTGCGGAATTGTTCGGT	construct generation
1326KpnIR1	ggtaccTGTAATGTTAGAGAATTGTATAGGAGT	construct generation
1326ApaIR1	gggcccCTCGACGGATACCACACCGGAGA	construct generation
1326SalIF1	gtcgacTAAtggtttcccgaagaacggtga	construct generation
SalIGFPR	gtcgacTTACTTGTACAGCTCGTCCATGCCGAGAGTGA	construct generation
CMycApaIF	gggcccGAA CAA AAA CTT ATT TCT GAA GAA GAT CTG TAAtggtttcccgaagaacggtga	construct generation
ApaIGFPF	gggcccGCTGCTGCCGCTGCCGCTGCGGC	construct generation



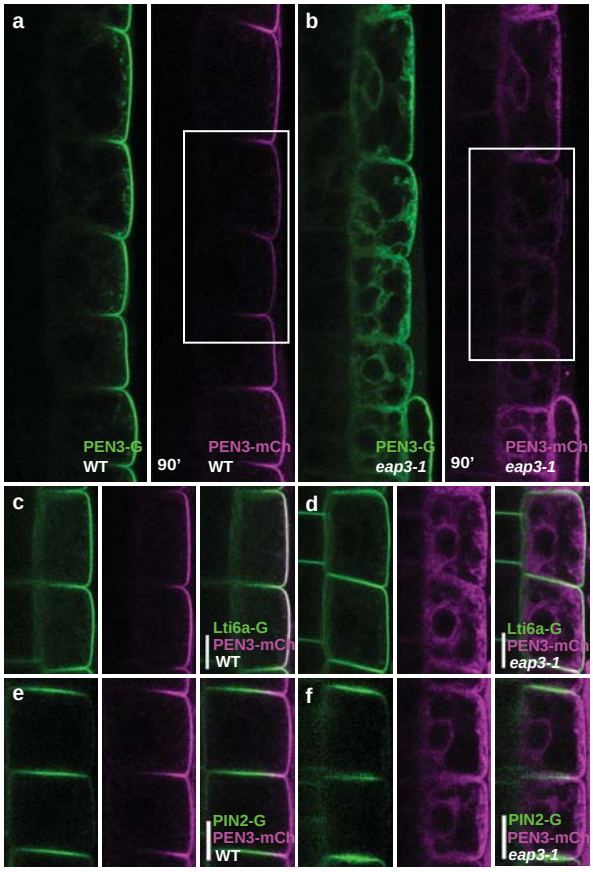
Supplementary Figure 1| Mislocalization of PEN3 to the ER in *eap3-2* and cytosolic localization of EAP3-GFP.
a,**b**, PEN3-GFP (PEN3-G; green) and PEN3-mCherry (PEN3-mCh; magenta) in wild type (**a**), *eap3-1* (**b**). **c**,**d**, PEN3-mCh (magenta) and anti-BIP immunolabelling (green) in Col-0 wild type expressing PEN3-mCh (**c**), and in *PEN3-mCherry eap3-1* mutant background (**d**). **e**,**f**, anti-BIP (green) immunofluorescence and PEN3-mCh (magenta) fluorescence in Col-0 wild type expressing PEN3-mCh (**e**), and in *PEN3-mCherry eap3-2* mutant background (**f**). **g**,**h**, PEN3-G localization in wild type (**g**), and in *eap3-1*/*eap3-2* transheterozygote (**h**). **i**, Cytosolic localization of EAP3-GFP. Note, no GFP signal in the nuclei. Arrowheads indicate selected nuclei. **j**, Col-0 wild-type control. BF, bright field images. Scale bars, 10 µm.



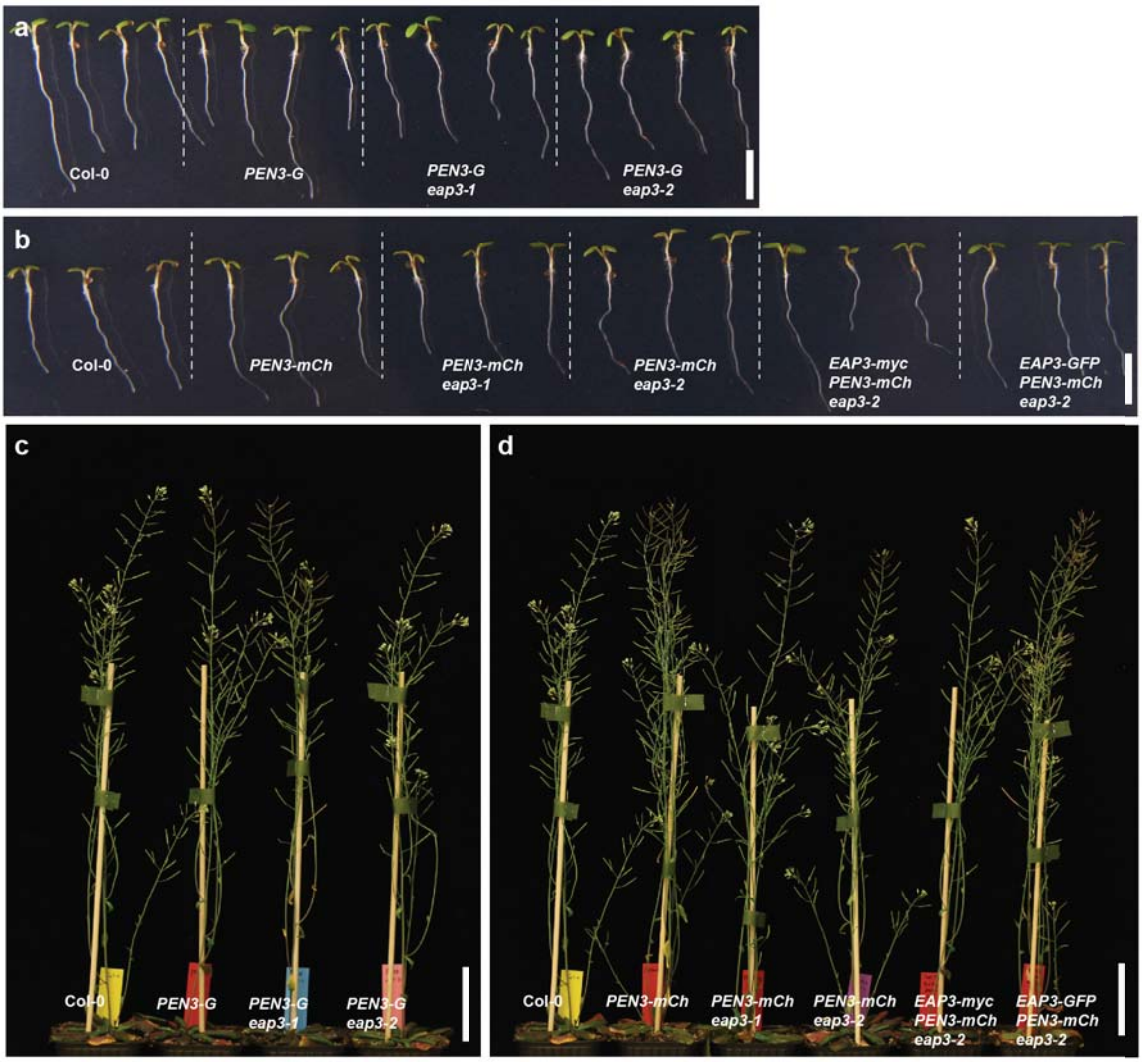
Supplementary Figure 2| Loss of weakly expressed EAP3 causes PEN3 protein accumulation.
a,b, Western blot of full-length EAP3 fusion proteins in protein extracts (input in (**a,b**) or after immunoprecipitation (IP) using Myc-Trap beads and anti-myc detection of EAP3-myc (55 kDa) in (**a**) or GFP-Trap_MA beads and anti-GFP detection of EAP3-GFP (78 kDa) in (**b**), in indicated genotypes. Asterisks indicate expected molecular weights of EAP3-myc (**a**), or EAP3-GFP (**b**). Note, unspecifically cross-reacting bands in protein extracts (input) versus single band of respective full-length protein in immunoprecipitations (IP). **c,d,** Western blot of PEN3-mCherry accumulation in indicated genotypes employing anti-RFP antibody (top) and anti-actin reference antibody (bottom). **e,f,** Quantitative and statistical analysis of relative PEN3-mCherry protein levels from experiments such as in (**c,d**). Error bars, SD from the average of three independent experiments (n = 3). Statistical differences were determined by Student's two-tailed t-test with equal variance. Significance level was $P < 0.05$. ** indicates $P < 0.01$. * indicates $P < 0.05$.



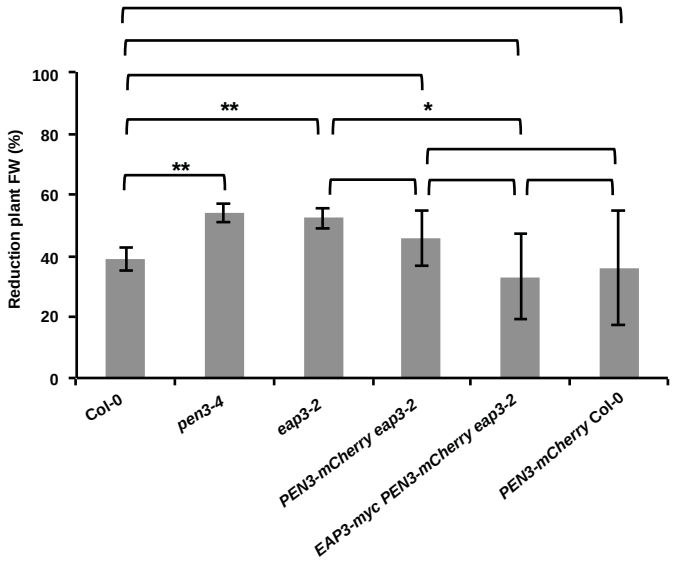
Supplementary Figure 3| Developmental map of transcript levels of *PEN3*, *EAP3* and *ACT7* genes. Absolute transcript levels (Transcript signal value) extracted from public databases using the eFP browser (refs. 32,33) are displayed employing “Absolute” mode and the “Developmental Map” as data sources with different developmental stages indicated. Error bars indicate SDs from averages of mostly triplicate experiments (*n* = 3) reported in ref. 33.



Supplementary Figure 4| EAP3 mediates PEN3 secretory trafficking from the ER with high cargo specificity.
a,**b**, Representative images for 90 min post-bleach displaying PEN3-mCherry (PEN3-mCh; magenta) and non-bleached PEN3-GFP (PEN3-G; green) reference in wild type (**a**) and *eap3-1* (**b**). Rectangular boxes indicate bleach ROIs in (**a**,**b**). **c**,**d**, LTI6a-GFP (Lti6a-G; green) and PEN3-mCh (magenta) in wild type (**c**) and *eap3-1* (**d**). **e**,**f**, PIN2-EGFP (PIN2-G; green) and PEN3-mCh (magenta) in wild type (**e**) and *eap3-1* (**f**).



Supplementary Figure 5| Comparison between phenotypes of wild type, *eap3* mutants, and rescue lines as seedlings and adult plants. a,**b**, Phenotypes of five-day-old seedlings. **a**, Wild type (Col-0), Col-0 expressing *PEN3-GFP* (*PEN3-G*), *PEN3-G eap3-1*, *PEN3-G eap3-2* that do not display obvious differences in overall seedling morphology. **b**, Col-0, Col-0 expressing *PEN3-mCherry* (*PEN3-mCh*), *PEN3-mCh eap3-1*, *PEN3-mCh eap3-2* and the rescue lines *EAP3-myc PEN3-mCh eap3-2* as well as *EAP3-GFP PEN3-mCh eap3-2* that all do not display obvious differences in overall seedling morphology. Scale bars, 5 mm. **c**,**d**, Phenotypes of eight-week-old plants. **c**, Wild type (Col-0), Col-0 expressing *PEN3-GFP* (*PEN3-G*), *PEN3-G eap3-1*, *PEN3-G eap3-2* that do not display obvious differences in overall adult plant morphology. **d**, Col-0, Col-0 expressing *PEN3-mCherry* (*PEN3-mCh*), *PEN3-mCh eap3-1*, *PEN3-mCh eap3-2* and the rescue lines *EAP3-myc PEN3-mCh eap3-2* as well as *EAP3-GFP PEN3-mCh eap3-2* that all do not display obvious differences in overall adult plant morphology. Scale bars, 5 cm.



Supplementary Figure 6| Effect of *F. oxysporum* inoculation on *pen3-4* and *eap3-2* mutants. Quantitative and statistical analysis of fresh weight (FW)-reduction displayed in % relative to the respective non-treated control of adult Arabidopsis plants of indicated genotypes 14 days after *F. oxysporum* inoculation. Averages from three independent experiments (n = 3) are shown with error bars indicating SD. Statistical differences of fresh weight reduction between genotypes were determined by Student's one-tailed t-test with equal variance (n = 3). The significance level is $P < 0.05$. ** indicates $P < 0.01$. * indicates $P < 0.05$. Exact P values are shown in Supplementary Table 1.