Supporting Information

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SI Materials and Methods

Molecular Biology. The cDNA of AP-1 μ-adaptin 2 (*AP1M2*) was obtained from the RIKEN Plant Science Center and isolated by PCR using primers AP1M2-forward (F) and AP1M2-reverse (R). Internal tags [myelocytomatosis viral oncogene homolog (myc) or 3×HA (hemagglutinin)] were introduced by a PCR approach using the primers shown in Table S3. Each tagging site is shown in Fig. S1. *AP1M2* with internal *myc* (*AP1M2:myc*) was ligated to the binary vector (XbaI/blunt end) *pCsV1300* with cassava vein mosaic virus promoter (*pCsVMV*) to generate *pCsVMV::AP1M2:myc*. For $AP1M2_{pro}$*::AP1M2:3×HA::*$AP1M2_{3UTR}$, a binary plasmid containing *AP1M2 cis*-regulatory sequences was generated as follows: a genomic fragment corresponding to the 3′ UTR region of *At1g60780* (approximately 0.3 kb) was PCR-amplified and subcloned into the *pGreenIIBASTA* plasmid (EcoRV/PstI). Next, a genomic fragment including the promoter and 5′ UTR of *At1g60780* (approximately 1.5 kb) was PCR-amplified and further subcloned into the *pGreenIIBASTA::*$AP1M2_{3UTR}$ plasmid (Acc65I/XhoI). PCR-amplified *AP1M2:3×HA* was inserted into the *pGreenIIBASTA::*$AP1M2_{pro}$*::*$AP1M2_{3UTR}$ plasmid (XhoI/EcoRV). For *35S::AP1M1:3×myc*, a *AP1M1* genomic fragment was isolated from the *Arabidopsis thaliana* genome and followed by insertion of the three times repeated myc epitope (BamH1) (F. El Kasmi, M. Werner, and G.J., Entwicklungsgenetik, ZMBP, University of Tübingen, Tübingen, Germany). *AP1M1:3×myc* was subcloned into the 35S expression vector (SmaI/XbaI). For *GST:α-adaptin*, *HA:α-adaptin* (1) was blunted after digestion with Acc65I and further ligated to the SmaI-cleaved pGEX-5X-3 plasmid (GE Healthcare). For *GST:γ-adaptin*, *γ-adaptin* was amplified by PCR using primers γ-F and γ-R and ligated to the pGEX-5X-1 (EcoRI/XhoI; GE Healthcare). For maltose-binding protein (*MBP*)*:AP1M2*, *AP1M2* was PCR-amplified using primers AP1M2-F2 and AP2M2-R2 and ligated to pMAL-c2X (BamHI/XbaI). For the list of primers used, see Table S3.

Plant Material, Growth Condition, and Transformation. *Arabidopsis* thaliana—ecotype *Columbia* (Col) or *Wassilewskija* (Ws)—plants were grown on soil or on Murashige and Skoog (MS) plates at 23 °C in continuous light. For protoplast preparation, *Arabidopsis thaliana* plants were grown on MS plates for 3 wk at 23 °C under long-day condition. Wild-type (WT) or heterozygous plants of either *ap1m2-1* [resistant to both kanamycin (KAN) and phosphinothricin (PPT); purchased from the Versailles Arabidopsis Stock Center] and or *hap13* (resistant to phosphinothricin) (2) were transformed with *Agrobacterium tumefaciens*, using the floral-dip method (3). GFP:*ARA7* (4) and PIN-FORMED 2 (PIN2):GFP (5) transgenic plants were crossed with *AP1M2::AP1M2:3×HA* transgenic plants or heterozygous plants of *ap1m2-1*.

Genetic Analysis. T1 plants grown on soil from bulk-harvested seeds were selected for transformants either with a 1:1,000 diluted BASTA (183 g/L glufosinate; AgrEvo) or with hygromycin (20 mg/L; Duchefa). Selected resistant plants were genotyped. PCR genotyping was as follows: for *ap1m2-1*, FLAG-LB4 and RP for a T-DNA insertion, amplifying a 0.7-kb fragment, and LP and RP for an endogenous *AP1M2*; for *hap13*, LB1a and RP-h13 for a T-DNA insertion, amplifying a 0.9-kb fragment, and LP and RP-h13 for an endogenous *AP1M2*; for *AP1M2:3×-HA*, primers HAs and M2-as, which amplify a 1-kb fragment; for *AP1M2:myc*, primers mycs and M2-as, which amplify a 0.8-kb fragment; and for *AP1M1:3×myc*, primers mycs and M1-as, which amplify a 0.8-kb fragment. Genomic DNA was isolated using a cetyltrimethylammonium bromide-based miniscale protocol as reported previously (6). For seedling observation, seeds were germinated on solid medium [2.15 g/L MS salts, 1 g/L MES, 1% (wt/vol) sucrose (pH 5.6)] in the same growth condition as described above. Segregation of antibiotics resistance was counted on PPT-supplemented (15 mg/L) and/or KAN-supplemented (30 mg/L) medium.

Transient Expression and in Vivo Targeting of Reporter Cargo Proteins. Plasmid DNA was introduced into protoplasts from leaf tissues by the polyethylene glycol-mediated transformation method as described previously (7). The expression and trafficking of GFP-fused cargoes, vacuolar sorting receptor (VSR)1:HA (8), and HA:δ-adaptin (1) were examined by Western blot analysis or by confocal fluorescence microscopy at different time points.

Western Blot Analysis and Coimmunoprecipitation. For immunoblot analysis, protein extracts were prepared with lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100] supplemented with an EDTA-free protease inhibitors mixture (Roche Diagnostics). Antibodies of anti-GFP (rabbit; 1:1,000; Clontech), anti-binding protein (BiP) (rabbit; 1:3,000) (8), anti-tubulin (mouse; 1:5,000; Sigma-Aldrich), anti-myc (1:1,000; Cell Signaling), anti–γ-adaptin (rabbit; 1:1,000), anti–α-adaptin (rabbit; 1:1,000) (9), or anti–γ-subunit of coatmer complex (γCOP) (rabbit; 1:2,000; Agrisera), anti-VSR (rabbit, 1:1,000) (8), anti-actin (MP Biomedicals), and peroxidase (POD)-conjugated anti-HA (1:1,000, Roche Diagnostics) were used to detect the indicated proteins. POD-conjugated secondary antibodies (1:7,000, Sigma-Aldrich) were used in all immunoblot analyses. Membranes were developed using a chemiluminescence detection system (Fusion Fx7 Imager; PEQlab) or an LAS3000 image capture system (Fujifilm).

For coimmunoprecipitation experiments, protein extracts from protoplasts were prepared in immunoprecipitation buffer [20 mM Hepes (pH 7.5), 125 mM potassium acetate, 2.5 mM magnesium acetate $Mg(OAc)_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1% Triton X-100, 0.1% NP-20, 1 mM protease inhibitor mixture]. Protein extract was incubated with anti-myc (Cell signaling technology) or anti-T7 (Novagen) antibody at 4 °C for 3 h and followed by incubation with protein A–agarose beads (Repligen) for 1 h, except for the experiment shown in Fig. S6*C*, in which protein G–agarose was used instead. The beads were washed three times with immunoprecipitation buffer and subjected to Western blot analysis.

Cell Fractionation. Total protein was extracted from WT (Ws) and *ap1m2-1* mutant seedlings, using a fractionation buffer [20 mM Hepes (pH 7.5), 125 mM potassium acetate, 2.5 mM $Mg(OAc)_2$, 125 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM protease inhibitor mixture]. After discarding cell debris, the supernatant (approximately 15 mg) was ultracentrifuged at 195,000 × *g* for 1 h at 4 °C. Resulting soluble (supernatant) and membrane fractions were subjected to immunoblot analysis.

Pull-Down Assay. Recombinant proteins GST:γ-adaptin and MBP:AP1M2 were purified from BL21(DE3) bacterial cells, using glutathione–agarose beads (Thermo Scientific) and amylase resin (New England Biolabs), respectively, and further incubated with protein extracts from seedlings, using pull-down buffer [20 mM Hepes (pH 7.5), 125 mM potassium acetate, 2.5 mM $Mg(OAc)_2$, 1 mM EDTA, 1 mM EGTA, 0.1% Triton X-100, 0.1% NP-20, 1 mM PMSF, protease mixture]. The beads were washed three times with the same volume of pull-down buffer and subjected to immunoblot analysis.

Semiquantitative RT-PCR Analysis of Transcripts. To determine the transcript level in the mutant plants, total RNA was isolated from the 7-d-old seedlings grown on plates using an RNeasy Mini Kit (Qiagen). Total RNA (2 μg) was reverse-transcribed using SuperScript II (Invitrogen) with random primers mixed with oligo (dT) primers. Equal amount of cDNA was used for PCR with gene specific primers listed in Table S3. PCR products were analyzed by ethidium bromide staining.

Anti–γ-Adaptin Antibody Generation. To generate anti–γ-adaptin antibody, the full-length γ-adaptin was expressed as a fusion protein with MBP using the pMAL-c2 vector (New England Biolabs). Recombinant MBP:γ-adaptin was expressed in *E. coli* BL21(DE3) strain and purified using amylose resin (New England Biolabs). MBP:γ-adaptin was used to immunize rabbits and antibody was affinity-purified using recombinant proteins. To confirm the specificity of the anti–γ-adaptin antibody, protein extracts from protoplasts transformed with *HA:γ-adaptin* were used in Western blot analysis (1).

Immunohistochemistry. Immunostaining of root tissues was performed as reported previously (10). In brief, 5-d-old seedlings were fixed in 4% (wt/vol) paraformaldehyde and labeled with the indicated antibodies. For immunofluorescence, anti-HA (mouse; 1:1,000; BAbCO), anti-γCOP (rabbit; 1:600; Agrisera), anti-syntaxin of plants 61 (SYP61) (rabbit; 1:600; a gift from N. Raikhel, University of Califonia Riverside, Riverside, CA), anti-clathrin heavy chain (CHC) (rabbit; 1:1,000) (11), anti-KNOLLE (rabbit; 1:4,000) (10), anti-tubulin (rat; 1:1,000; Abcam), or anti-PIN2 (rabbit, 1:600) (5) antibodies were used. As the secondary antibody, FITC-labeled anti-mouse IgG (1:600; Dianova), Cy3-labeled anti-rabbit IgG (1:600; Dianova), Alexa488-labeled anti-rabbit IgG (1:600; Invitrogen), or FITC-labeled anti-rat IgG (1:600; Dianova) were used.

Chemical Treatment. Five-day-old seedlings were treated with 50 μM brefeldin A (BFA) (50 mM stock solution in 1:1 DMSO/EtOH; Invitrogen) or 20 μM wortmannin (20 mM stock solution in DMSO) for 1 h, followed by fixation or immediate observation. For FM4-64, 2 μM FM4-64 was added to 5-d-old seedlings (4 mM stock solution in H_2O; Invitrogen). For simultaneous treatment with BFA and FM4-64, BFA was followed by addition of FM4-64. For BFA washout, BFA-treated seedlings for 1 h were transferred to new liquid medium and fixed 30 min after the transfer. For concanamycin A (ConcA) treatment, protoplasts isolated from WT seedlings were treated at indicated concentrations (1 mM stock solution in DMSO, Invitrogen). For cycloheximide (CHX) treatment, 50 μM CHX was added to seedlings for 1 h (50 mM stock solution in H_2O; Sigma-Aldrich).

Ultrastructure Analysis. Root tips of 5-d-old seedlings were high-pressure-frozen in 1-hexadecene (Merck Sharp and Dohme) using a Bal-Tec HPM 010 high-pressure freezer (Balzers). Frozen samples were freeze-substituted in acetone containing 2.5% (wt/vol) osmium tetroxide (2 d at −90 °C, 6 h at −60 °C, 6 h at −30 °C, and 1 h at 0 °C) and finally embedded in epoxy resin (Roth). Ultrathin sections were stained with 2% (wt/vol) uranyl acetate in 50% ethanol for 10–20 min and lead citrate (3–5 min).

Software. Sequences were analyzed using Vector NTI (Invitrogen) or CLC DNA Workbench 5. Images were processed using Adobe Photoshop CS3 and Adobe Illustrator CS3. Quantification of confocal laser scanning microscopy (CLSM) images was done using Pearson and Spearman correlation coefficients (PSC) colocalization plugin of ImageJ [National Institutes of Health (NIH)] after adjusting the background level to 5 in five independently taken images according to instructions in the paper by French et al. (12). Signal intensity in immunoblot analyses were determined using ImageJ (NIH). Pixel count in live seedlings was done using Leica LAS in three or four independently taken images.

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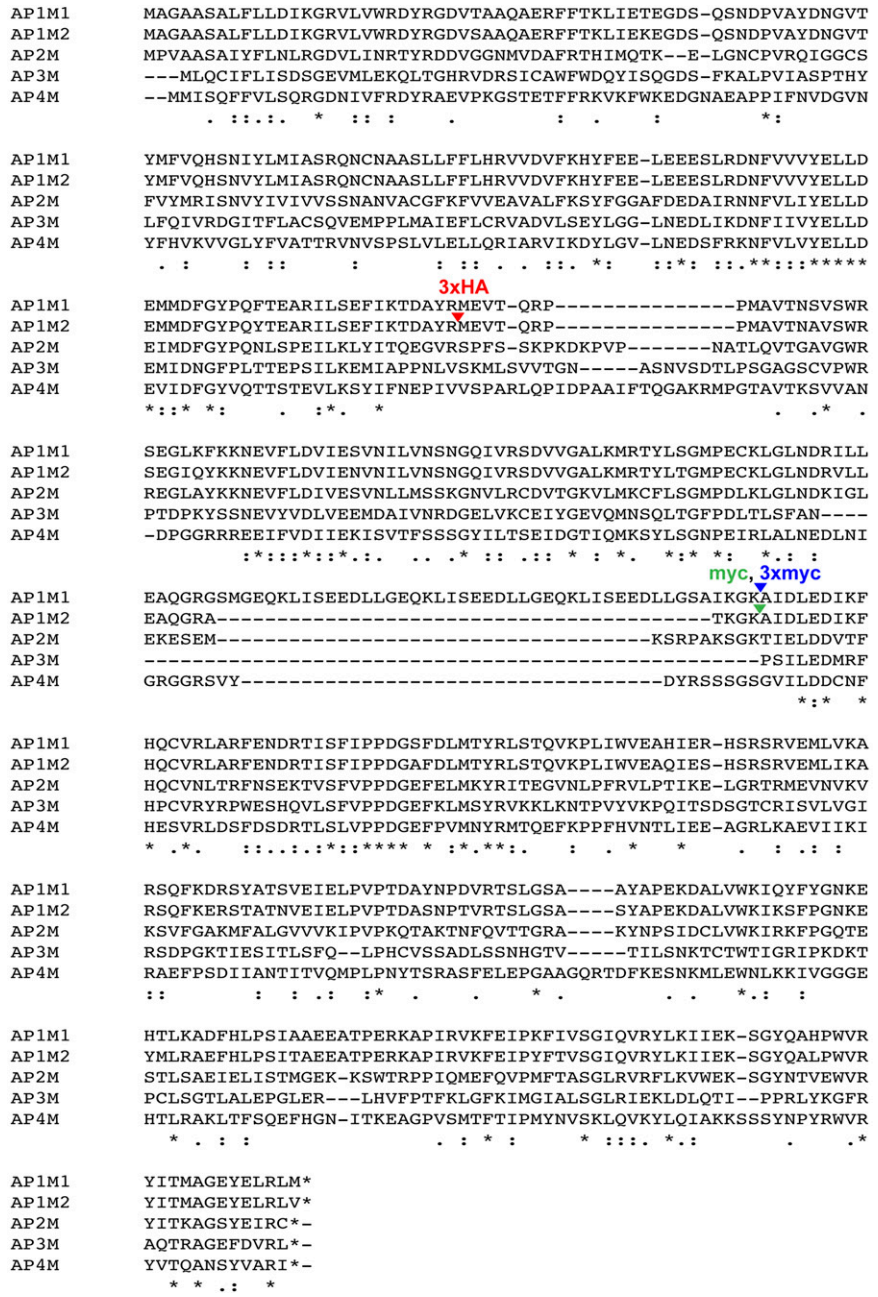

Fig. S1. Alignment of amino acid sequences of the *Arabidopsis* mu adaptins. Amino acid sequences of AP1M1 (At1g10730), AP1M2 (At1g60780), AP2M (At5g46630), AP3M (At1g56590), and AP4M (At4g24550) were compared using CLUSTAL Omega. Note that AP1M1 and AP1M2 share 90% sequence identity (analyzed in CLUSTAL Omega). Red and green triangles indicate the insertion sites of three times repeated HA (3×HA) and myc epitope in AP1M2, respectively. Blue triangle indicates the insertion site of three times repeated myc (3×myc) epitope in AP1M1.

A

	ap1m2-1 %	KAN^R %	KAN^S %	N
Self-fertilized *ap1m2-1/AP1M2*	9	53	47	992
ap1m2-1/AP1M2 (♀) x *Ws* (♂)	-	48	52	302
Ws (♀) x *ap1m2-1/AP1M2*(♂)	-	8	92	954

B

	hap13 %	PPT^R %	PPT^S %	N
Self-fertilized *hap13/AP1M2*	5	52	48	232
hap13/AP1M2 (♀) *x Col* (♂)	-	55	45	100
Col (♀) *x hap13/AP1M2* (♂)	-	6	94	96

C

F1 plants		F2 seedlings	
Transgenes	allele	*ap1m2-1* (%)	N
AP1M2:myc #1	*ap1m2-1/AP1M2*	2	75
AP1M2:myc #2	*ap1m2-1/AP1M2*	2	105
AP1M2:3xHA #1	*ap1m2-1/AP1M2*	2	287
AP1M2:3xHA #4	*ap1m2-1/AP1M2*	2	265

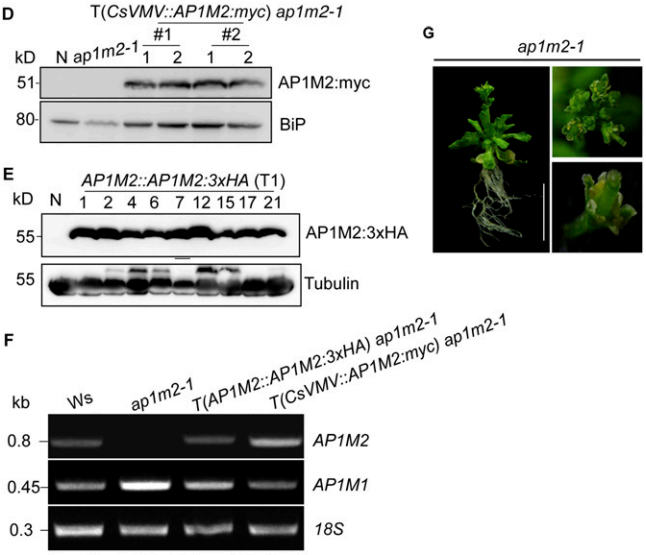

Fig. S2. Genetic analysis of T-DNA insertional *AP1M2* mutants and phenotype of mature *ap1m2-1* mutant grown in a closed container. (*A* and *B*) Reciprocal crosses of *ap1m2* heterozygotes with the respective WT (Ws, Wassilewskija or Col, Columbia) revealed reduced transmission of *ap1m2* mutant alleles *ap1m2-1* [KAN resistance (KAN^R)] (*A*) or *hap13* [PPT resistance (PPT^R)] (*B*) through the pollen, resulting in only 9% or 5% *ap1m2* homozygous seedlings upon selfing of heterozygotes, respectively. N, total number of seedlings analyzed. (*C*) Complementation tests. Transgenic plants each carrying a different single T-DNA insertion of *CsVMV::AP1M2:myc* (Hyg^R) or *AP1M2::AP1M2:3×HA* (PPT^R) were crossed with *ap1m2-1* heterozygous plants. F2 progenies (N, number of seedlings) were analyzed for the phenotypic segregation and genotyped. Note that 2% *ap1m2-1* homozygous seedlings corresponds to the expected value if 75% of approximately 9% *ap1m2-1* homozygous seedlings from selfed *ap1m2-1* heterozygous plants are rescued by the transgene (*A*). R, resistant; S, sensitive. (*D* and *E*) Immunoblot analysis of CsVMV::AP1M2:myc and AP1M2::AP1M2:3×HA plants. (*D*) Two independent lines of *CsVMV::AP1M2:myc* harboring a single T-DNA insertion were crossed with *ap1m2-1* heterozygous plants. Total protein of nontransformed (N), *ap1m2-1* homozygous, and rescued seedlings was subjected to immunoblot analysis with anti-myc or anti-BiP antiserum. T, transgene. (*E*) Total protein of nontransformed (N) and T1 plants harboring *AP1M2::AP1M2:3×HA* was subjected to immunoblot analysis with anti-HA or anti-tubulin antiserum. Plants bearing a single T-DNA insertion (#1 and #4) were crossed with the *ap1m2-1* heterozygous plants for complementation test (*C*). Molecular mass markers are shown at the left. (*F*) Semiquantitative RT-PCR analysis to compare the level of transgenically expressed epitope-tagged *AP1M2* in rescued mutant lines to that of endogenous *AP1M2* in WT. Note that the level of *AP1M2:3×HA* and *AP1M2:myc* is comparable to that of WT. *18S rRNA* (*18S*) was used as loading control. Molecular mass markers are shown at the left. (*G*) Phenotype of mature *ap1m2-1* mutant grown in a closed container. Note that the mutant presents ill-developed anthers. (Scale bar: 1 cm.)

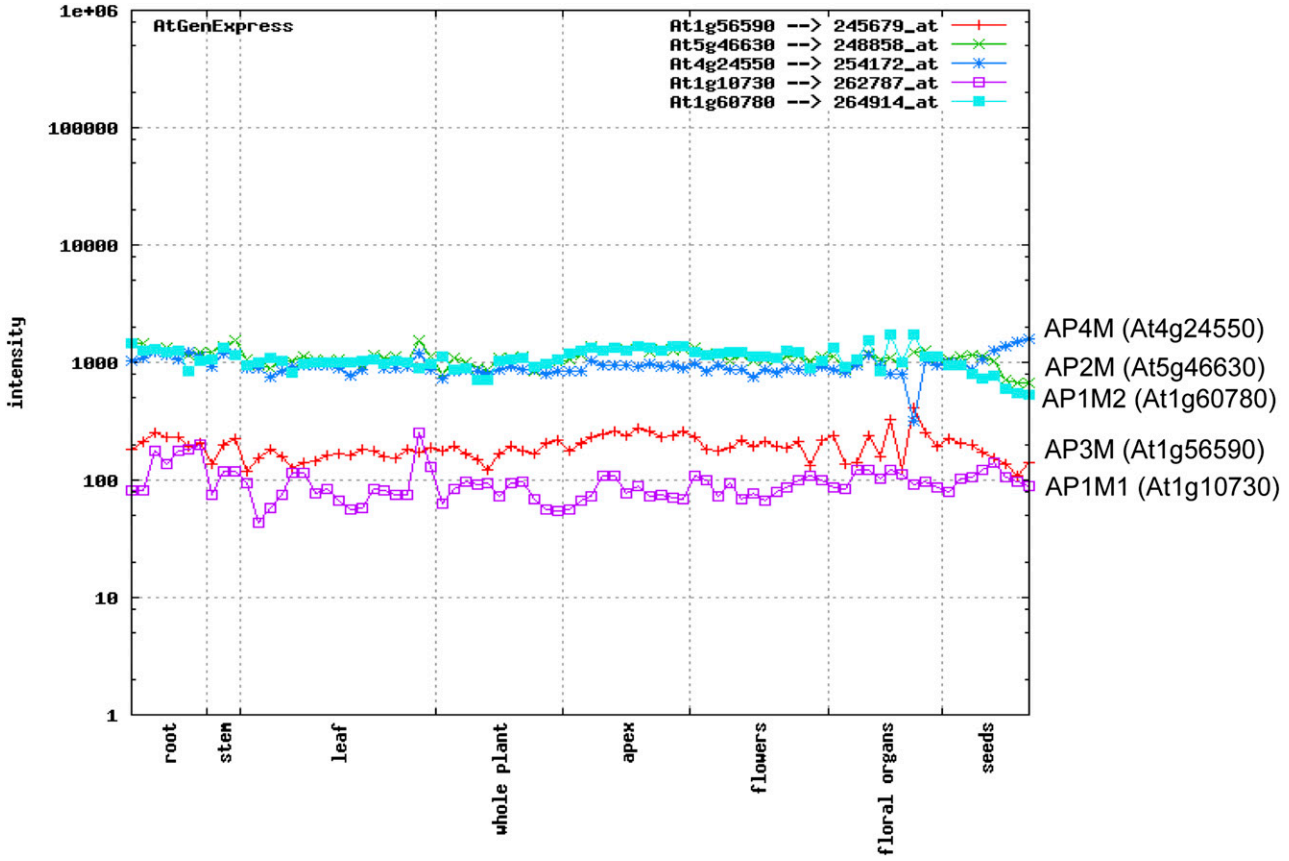

Fig. S3. Expression profile of the *Arabidopsis* mu adaptins. Expression levels of AP1M1 (magenta), AP1M2 (turquoise), AP2M (green), AP3M (red), and AP4M (blue) were compared using the AtGenExpress Visualization Tool (www.weigelworld.org/resources/microarray/AtGenExpress) (1).

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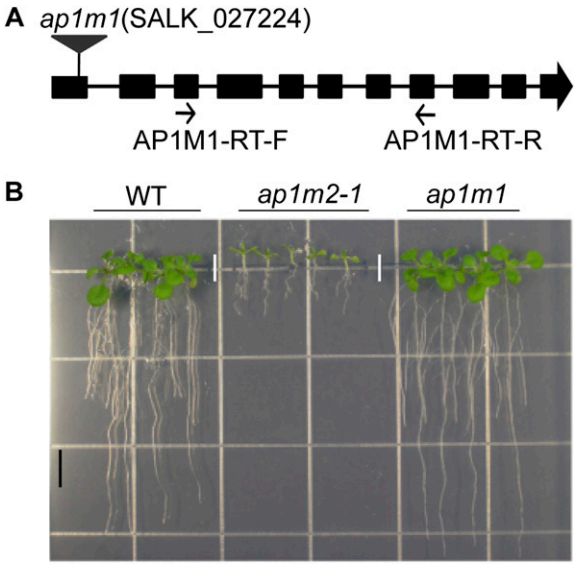

Fig. S4. T-DNA insertion *AP1M1* mutant does not show any discernible morphological change. (*A*) Diagram of the T-DNA insertion in *AP1M1*, *ap1m1* (SALK_027224). Primers used in Fig. 1*E* are indicated. (*B*) Images of *ap1m1* homozygous seedlings grown on an MS plate for 7 d. Note that seedlings of the *ap1m1* mutant look normal, in contrast to the *ap1m2-1* mutant. (Scale bar: 1 cm.)

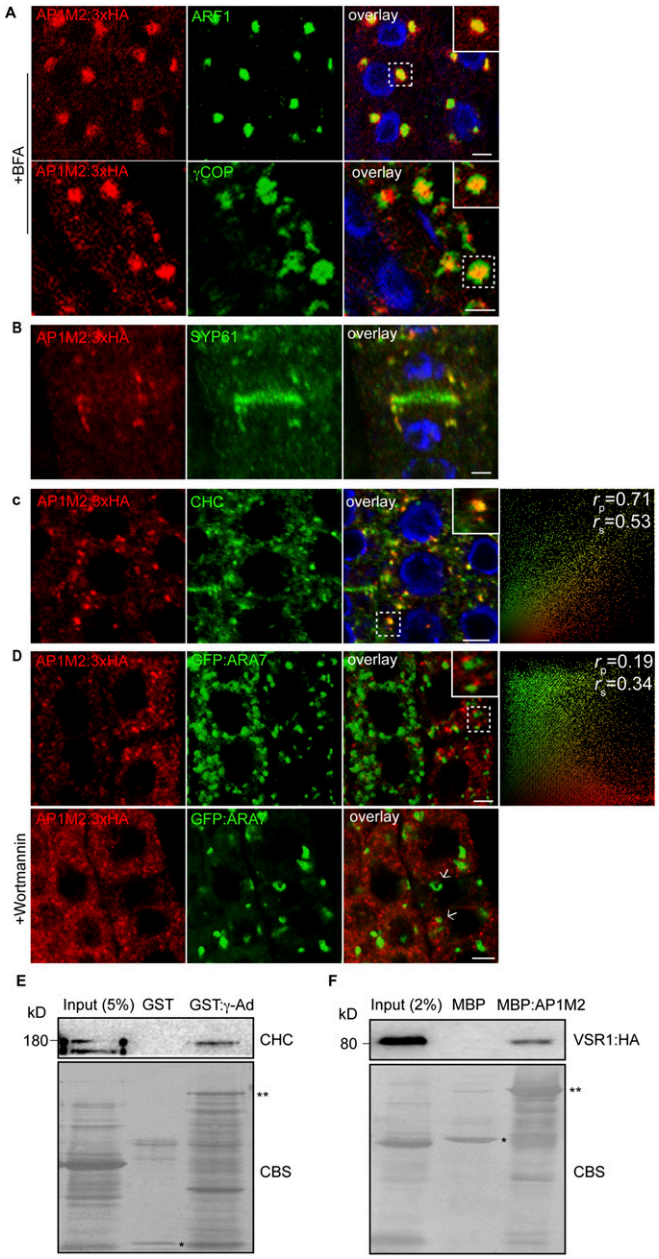

Fig. S5. Localization of tagged AP1M2 in seedling roots and the interaction of γ-adaptin–containing AP1 complex with clathrin and VSR. (*A*) After BFA treatment, AP1M2:3×HA in *ap1m2-1* homozygous background was counterstained with anti-ARF1 (*Upper*) or anti-γCOP (*Lower*) antiserum. Note that AP1M2:3×HA colocalized with ARF1 in endosomal BFA compartments surrounded by γCOP-positive Golgi stacks. (*B* and *C*) AP1M2:3×HA in *ap1m2-1* homozygous background was counterstained with anti-SYP61 (*B*) or anti-CHC (*C*) antiserum. Note that the tagged AP1M2 does not accumulate at the cell plate, which is positively labeled with anti-SYP61 antiserum (*B*). Note partial colocalization of AP1M2:3×HA with clathrin (*C*). (*D*) AP1M2:3×HA and the multivesicular body (MVB) marker GFP:ARA7/RAB-F2b were examined for colocalization. AP1M2:3×HA was detected by immunostaining with anti-HA antibody and GFP signals of GFP:ARA7/RAB-F2b were observed directly. AP1M2:3×HA-positive endosomes were insensitive to wortmannin, in contrast to the swollen ARA7/RabF2b-labeled prevacuolar compartment/multivesicular body/late endosome (PVC/MVB/LE) (white arrows). The extent of colocalization was quantified using the linear Pearson correlation coefficient (r_p) and the nonlinear Spearman rank correlation coefficient (r_s), and the resulting scatterplot images are shown on the right (*C* and *D*). A value of 1.0 means complete colocalization of two fluorescent signals. Note partial colocalization of AP1M2:3×HA with clathrin, as quantified by r_p/r_s = 0.71/0.53 and no localization of AP1M2:3×HA at the PVC/MVB labeled with GFP:ARA7/RAB-F2b, as indicated by r_p/r_s = 0.19/0.34. *Insets* show boxed areas at higher magnification. (Scale bars: 5 μm.) (*E*) Purified recombinant GST or GST:γ-adaptin protein was precipitated with glutathione beads after incubation with plant extracts from WT seedlings and followed by immunoblot analysis. Note that CHC is detected in the precipitate but not in the control precipitate done with GST alone. The Coomassie-stained membrane (CBS) showed bands of GST (asterisk) and GST:γ-adaptin (double asterisks). (*F*) Pull down of HA-tagged VSR1 from protoplasts, using recombinant MBP fused to AP1M2; MBP alone was used as control. The CBS showed bands of MBP (asterisk) and MBP:AP1M2 (double asterisks). Molecular mass markers are indicated in kilodaltons (kDa).

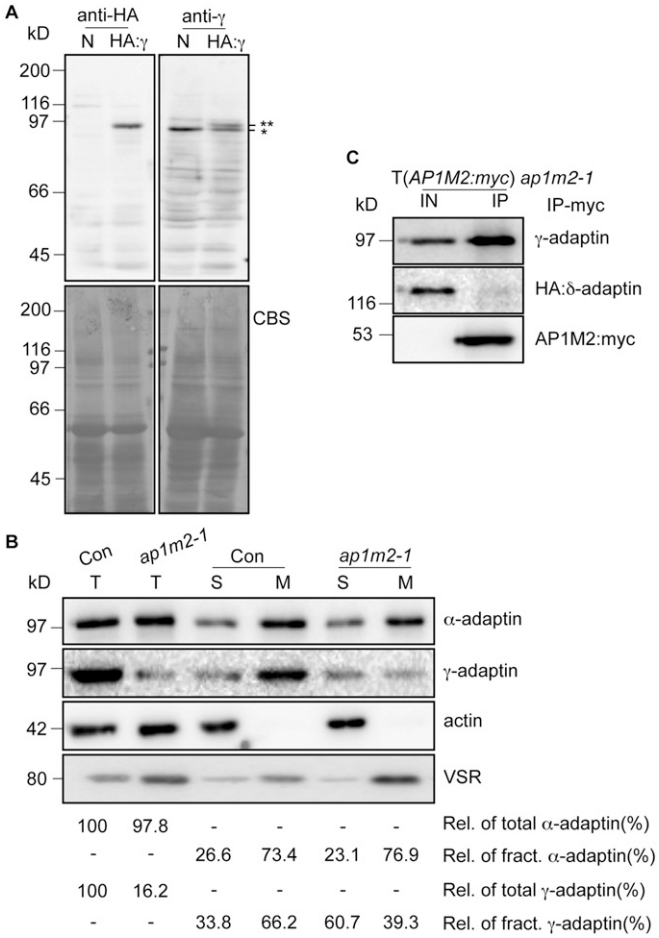

Fig. S6. Specificity determination of anti–γ-adaptin antiserum and no interaction of AP1M2 with δ-adaptin. (*A*) Total protein extracts from protoplasts transformed with *HA:γ-adaptin* (HA:γ) were analyzed by immunoblotting using anti-HA antibody and anti–γ-adaptin antiserum. N, nontransformed protoplasts. Asterisk, endogenous γ-adaptin; double asterisks, transiently expressed HA:γ-adaptin. *A*, *Lower* shows the CBS. (*B*) Fractionation analysis. Total protein was extracted from WT (Con) or *ap1m2-1* mutant seedlings, followed by microultracentrifugation. Note that total protein level of γ-adaptin in *ap1m2-1* mutant is highly reduced by approximately 80% compared with the WT and that less γ-adaptin is detected in the membrane (M) fraction of the *ap1m2-1* mutant compared with WT. Actin and VSR were detected as controls of soluble (S) and membrane fractions, respectively. Rel. of adaptins, relative signal intensity: total (T) signal of *ap1m2*-1 is expressed as percentage of control (Con, WT; 100%). The relative signal intensity of soluble and membrane fraction in each case is expressed as percentage of the total signal. (*C*) Protein extract [input (IN)] from rescued protoplasts (*CsVMV::AP1M2:myc* in *ap1m2-1* mutant background) that had been transformed with *35S::HA*:δ-*adaptin* was subjected to immunoprecipitation (IP) with anti-myc antibody and the immunoprecipitate was analyzed by immunoblotting using anti-HA, anti-myc, or anti–γ-adaptin antiserum. Note that HA:δ-adaptin is not immunoprecipitated with AP1M2, in contrast to γ-adaptin. Molecular mass markers are indicated in kilodaltons (kDa).

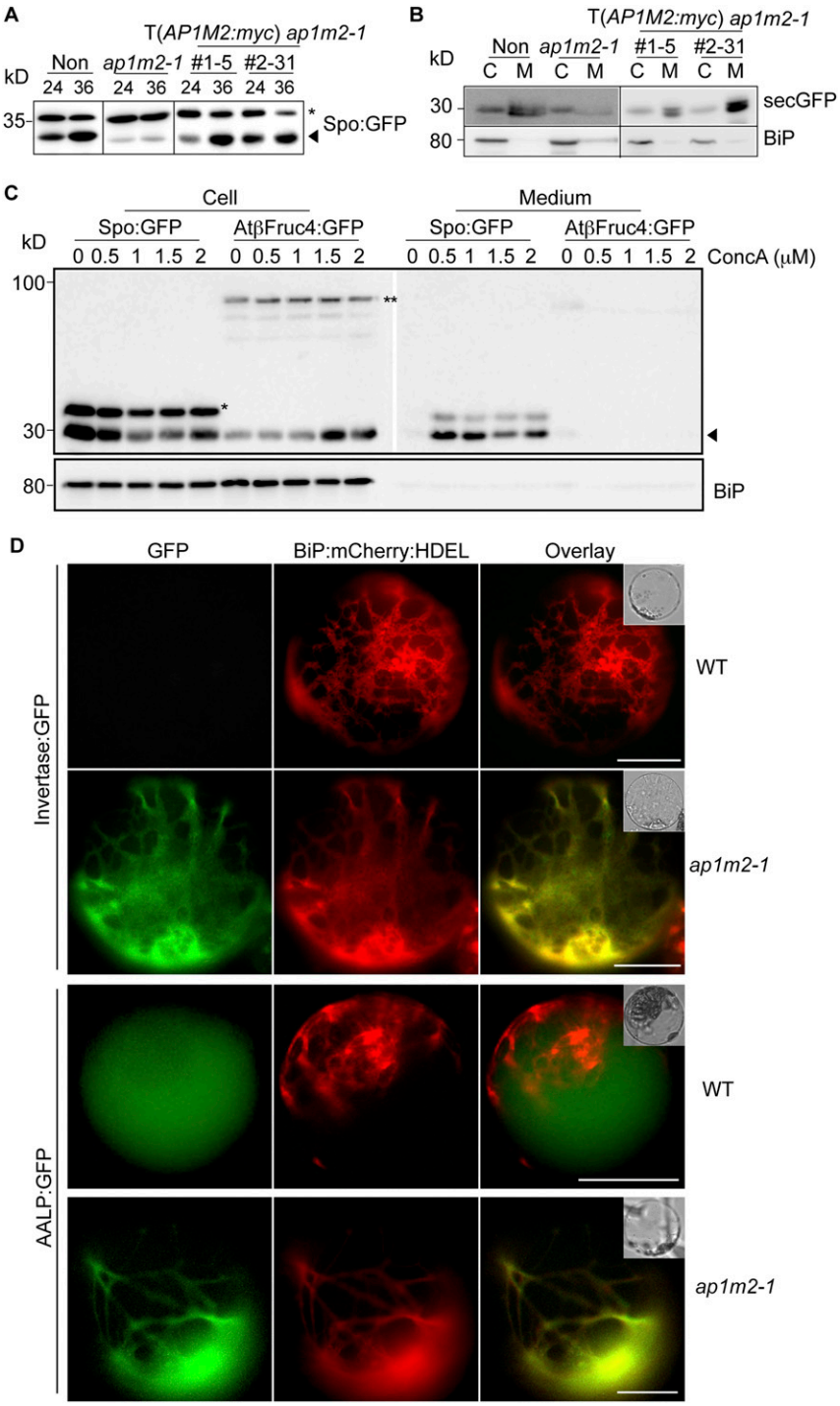

Fig. S7. Vacuolar and secretory pathways are impaired in the *ap1m2* mutants. (*A*) Trafficking of vacuolar cargo. Protoplasts from nontransformed, *ap1m2-1*, and two independent rescued plants (*CsVMV::AP1M2:myc* in *ap1m2-1*) were transformed with *Sporamin:GFP (Spo:GFP)*. At 24 or 36 h after transformation, total protein extracts from protoplasts were subjected to immunoblot analysis with anti-GFP antiserum. Asterisk, full-length Spo:GFP; closed triangle, processed Spo:GFP. (*B*) Trafficking of secretory cargo. Secretory GFP (*secGFP*) was transformed into protoplasts from the indicated plants. At 24 h after transformation, total protein extracts from protoplasts (C) and the incubation medium (M) were subjected to immunoblot analysis with anti-GFP antiserum. BiP was used as a control for nonspecific leaking of cellular proteins. (*C*) Protoplasts transformed with *Spo:GFP or AtβFructosidase4:GFP* (*AtβFruc4:GFP*) were treated with ConcA at the indicated concentrations. At 24 h after transformation, total protein extracts from protoplasts were subjected to immunoblot analysis with anti-GFP antiserum. Asterisk, full-length Spo:GFP; double asterisks, full length AtβFruc4:GFP; closed triangle, processed forms of AtβFruc4:GFP and Spo:GFP. Note that ConcA treatment inhibits the delivery of Spo:GFP, but not of AtβFruc4:GFP, to the vacuole. Both precursor and mature Spo:GFP are secreted from ConcA-treated protoplasts into the medium. Endoplasmic reticulum (ER)-localized BiP was used as control for intact cells. (*D*) Localization of soluble vacuolar and secretory proteins in *ap1m2-1* mutant protoplasts. Protoplasts from WT or *ap1m2-1* mutant plants were transformed with the indicated constructs and subcellular localization of the proteins was examined. Note that AALP:GFP and invertase:GFP accumulate in the ER, as indicated by their colocalization with BiP:mCherry:HDEL in the *ap1m2-1* protoplasts. (Scale bars: 10 μm.)

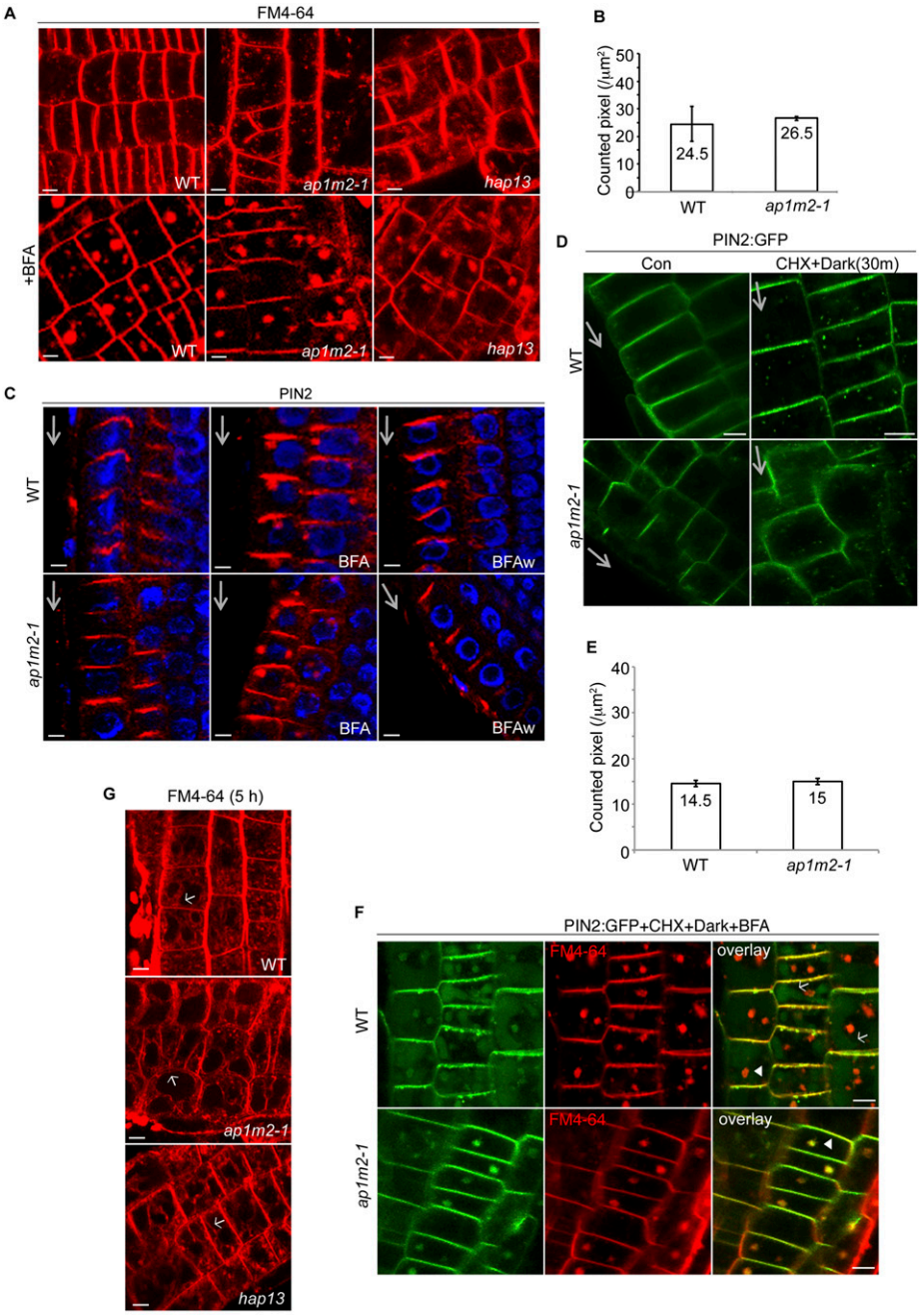

Fig. S8. Endocytosis and recycling of PIN2 occur normally in the *ap1m2* mutants but vacuolar traffic of PIN2:GFP is impaired in the mutant. (*A* and *B*) No obvious defect in endocytosis in *ap1m2-1* mutant plants. (*A*) WT, *ap1m2-1*, and *hap13* mutant seedlings were treated with FM4-64 alone or together with BFA. Note intracellular FM4-64–positive endosomes in the seedling roots of all three genotypes. As in WT, BFA compartments were observed in *ap1m2* seedling roots after BFA treatment, indicating that endocytosis of FM4-64 occurs normally in *ap1m2* mutants. (*B*) Measurement of FM4-64 internalization in WT and *ap1m2-1* seedling roots. Pixels of intracellular FM4-64 signal taken 5 min after uptake were counted from three different images. The mean value of counted pixels per square micron was calculated. Error bars indicate SD. (*C*) Normal recycling of PIN2 in *ap1m2-1* plants. WT and *ap1m2-1* homozygous seedlings were fixed and stained with anti-PIN2 antiserum. For BFA washout (BFAw), seedlings were incubated in BFA-free medium for 30 min after 1 h BFA treatment. Note the disappearance of the BFA compartment and restoration of PIN2 polar localization at the plasma membrane after BFA washout, indicating that PIN2 recycling occurs normally in *ap1m2-1* seedling roots compared with WT roots. Gray arrows indicate the direction of gravity. (*D* and *E*) Measurement of PIN2:GFP internalization in *ap1m2-1* mutant seedling roots. PIN2:GFP was observed after incubating 4-d-old seedlings with 50 μM CHX for 1 h, followed by darkness (Dark) for 30 min. Note appearance of PIN2:GFP-labeled intracellular endosomes in *ap1m2-1*, similar to the level in WT, which also presents weak, but discernible, vacuolar signals of PIN2:GFP. Control (Con) images show PIN2:GFP in WT or *ap1m2-1* under normal growth condition. (*E*) Measurement of PIN2:GFP internalization in WT and *ap1m2-1* seedling roots. Pixels of PIN2:GFP signal were counted from four different images. The mean value of counted pixels per square micron was calculated. Error bars indicate SD. (*F*) Confocal images showing impaired delivery of PIN2:GFP to the vacuole in *ap1m2-1*. Seedlings were treated with 50 μM CHX for 1 h followed by darkness (Dark) for 30 min and BFA treatment. Note that PIN2:GFP is observed in both the vacuole (indicated by arrows) and BFA bodies (indicated by arrowheads) in WT, whereas PIN2:GFP is mainly observed in BFA bodies in the *ap1m2-1* mutant. (*G*) No alteration of vacuolar morphology in the *ap1m2-1* and *hap13* mutants. Vacuolar tonoplasts were observed 5 h after application of FM4-64. Note normal-looking tonoplasts in the mutants. Blue, DAPI staining of chromatin in *C*. (Scale bars: 5 μm).

Table S1. Genetic analyses of *ap1m1 ap1m2* double mutants

Parental genotype	Unfertilized ovules, % (N)	Fertilized ovules, % (N)
Col	2 (5)	98 (185)
ap1m1/AP1M1 ap1m2-1/AP1M2	32 (206)	68 (416)
ap1m1/AP1M1 hap13/AP1M2	20 (143)	80 (591)

N, number of ovules analyzed in F1 progeny of self-fertilized plants.

Table S2. Genetic analyses of *ap1m1 ap1m2* double mutants

Female X male (parental genotypes)	PPT^R KAN^R, % (N)*	PPT^S KAN^S,% (N)†
Col X *ap1m1/AP1M1 ap1m2-1/AP1M2*	2 (4)	98 (182)
ap1m1/AP1M1 ap1m2-1/AP1M2 X *Col*	50 (82)	50 (82)
Col X *ap1m1/AP1M1 hap13/AP1M2*	0 (0)	100 (92)
ap1m1/AP1M1 hap13/AP1M2 X *Col*	26 (18)	74 (52)

N, number of seedlings analyzed in F1 progeny from crosses.
*Seedlings resistant to both PPT and KAN.
†Seedlings sensitive to either PPT or KAN.

Table S3. List of primers used

Genes	Primer name	Direction	Sequence (5′–3′)
Constructs			
AP1M2 cassette	AP1M2 promoter-5	Forward	ATTGCCCATGATTCATTTGTA
	AP1M2 promoter-3	Reverse	TTTTTTCTCGAGGTTCTTCCCTAAGAGTTTCGAT
	AP1M2 3′ UTR-5	Forward	AAAAAAGATATCTGCACATTTAGTTTCATCGTC
	AP1M2 3′ UTR-3	Reverse	TTTTTTCTGCAGGCCCCAAAAAACAAAACCA
AP1M2:3×HA*	M2-s	Forward	AAAAAACTCGAGATGGCAGGAGCTGCCTCC
	M-HA3	Reverse	AGGAACATCGTATGGGTACATCATTCTATATGCATCAGTCTT
	M-HA5	Forward	AAGACTGATGCATATAGAATGATGTACCCATACGATGTTCCT
	HA-M3	Reverse	TGGAGGTCTCTGTGTAACTTCAGCGTAATCTGGAACGTCATA
	HA-M5	Forward	TATGACGTTCCAGATTACGCTGAAGTTACACAGAGACCTCCA
	M2-as	Reverse	TTTTTTGGATCCTTACACGAGTCTTAGTTCGTA
AP1M2:myc†	AP1M2-F	Forward	CCCTCTAGAATGGCAGGGGCGGCCTCAGCG
	Internal-MYC-3	Reverse	ACTAAGGGAAAAGCCGAACAAAAGCTTATTTCTGAAGAAGAT CTTATTGATTTG
	Internal-MYC-5	Forward	GATGTCCTCCAAATCAATAAGATCTTCTTCAGAAATAAGCTTTT GTTCGGCTTTTCCCTT
	AP1M2-R	Reverse	GGGCTCGAGTTACACGAGTCTTAGTTCGTA
AP1M1:3xmyc	M1-s	Forward	AAAAAA CTCGAGATGGCAGGGGCGGCCTCA
	M1-as	Reverse	TTTTTTCCCGGGTTACATGAGTCTCAGTTCGTA
GST:g-Ad	γ-F	Forward	TGCTCTAGAATGAATCCCTTTTCTTC GGTA
	γ-R	Reverse	CCGCTCGAGTCACAACCCGCGAGGGAAGTTG
MBP:AP1M2	AP1M2-F2	Forward	CGCGGATC ATGGCAGGGGCGGCCTCAGCG
	AP1M2-R2	Reverse	TGCTCTAGATTACACGAGTCTTAGTTCGTA
Genotyping			
ap1m2-1 allele	FLAG_LB4	Forward	CGTGTGCCAGGTGCCCACGGAATAGT
	LP	Forward	ATGTCCTAACGGTGGGGTTAG
	RP	Reverse	CCGAAGCAAGAATTCTCAGTG
hap13 allele	LB1a	Forward	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTC
	RP-h13	Reverse	TTTTTTGGATCCTTACACGAGTCTTAGTTCGTA
ap1m1 allele	salk LB1.3	Forward	ATTTTGCCGATTTCGGAAC
	Salk_027224-LP	Forward	TAGTGACAGCCATTGGAGGTC
	Salk_027224-RP	Reverse	TTTCGCAGAATAATGCAAAGC
HA	HAs	Forward	GTTCCTGACTATGCGGGCTAT
myc	mycs	Forward	ATGGAGCAAAAGCTTATTTCT
RT-PCR			
AP1M2-RT-F		Forward	TCGAGGAGTTAGAAGAGGAACA
AP1M2-RT-R		Reverse	CGTTCGTTGCGGTGCTTCGT
AP1M1-RT-F		Forward	GAGGAGTGAGGGGTTAAAGTTT
AP1M1-RT-R		Reverse	TCAATCTCAACACTTGTTGCATA
18S RNA-F		Forward	ATGATAACTCGACGGATCGC
18S RNA-R		Reverse	CCTCCAATGGATCCTCGTTA

*AP1M2:3×HA was generated by a two-step PCR amplification: a fragment was amplified with primers M2-s and M-HA3 from AP1M2, a fragment with primers M-HA5 and HA-M3 from 3×HA and a fragment with primers HA-M5 and M2-as from AP1M2. The final PCR amplification of the three PCR products was done with primers M2-s and M2-as.

†AP1M2:myc was generated by a two-step PCR amplification: a N-terminal fragment was amplified with primers AP1M2-F and internal-MYC-3 from AP1M2 and a C-terminal fragment with primers internal-MYC-5 and AP1M2-R from AP1M2. Subsequent PCR was done with the two PCR products and primers AP1M2-F and AP1M2-R.