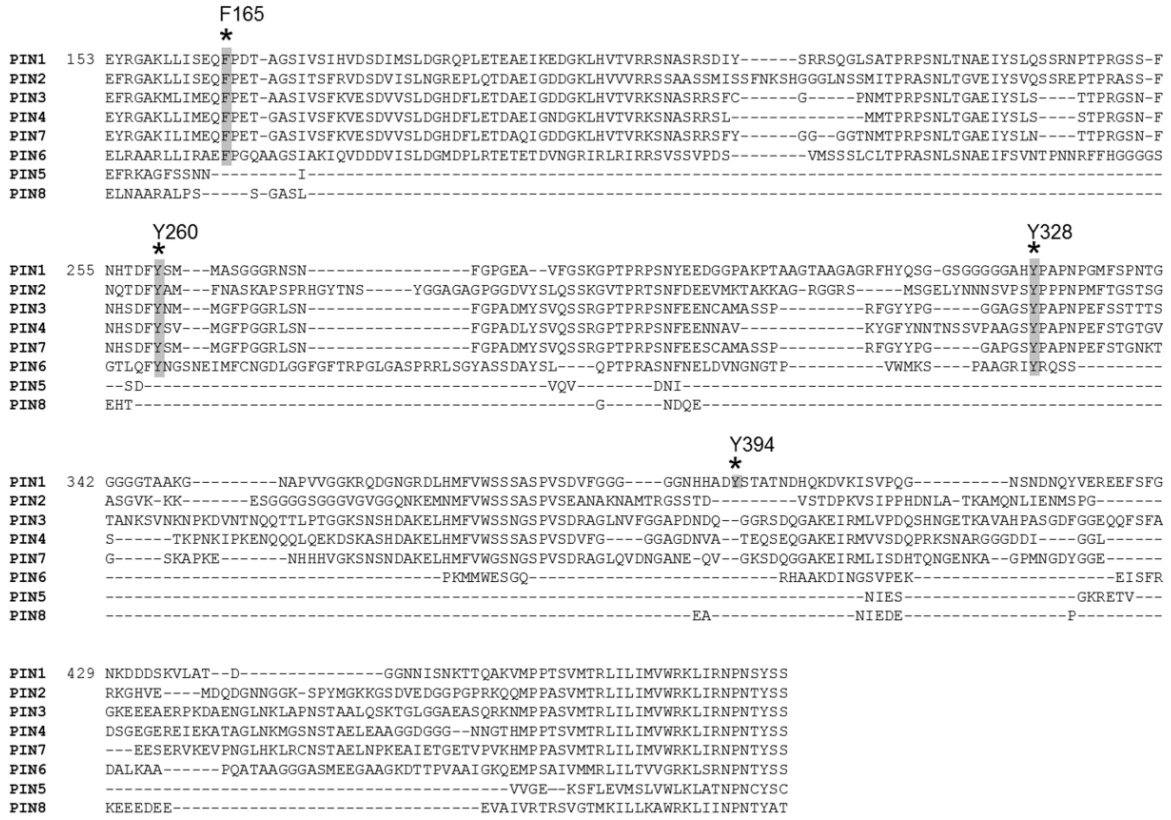
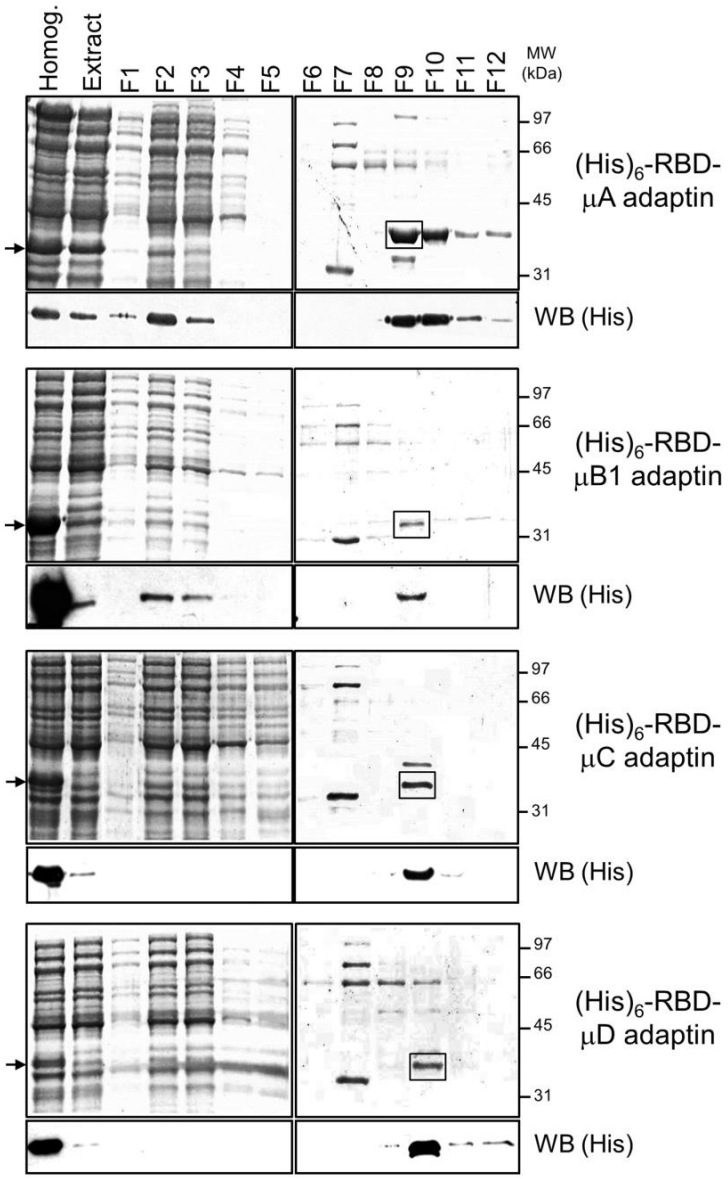
SUPPLEMENTAL MATERIAL

Supplemental Figure S1



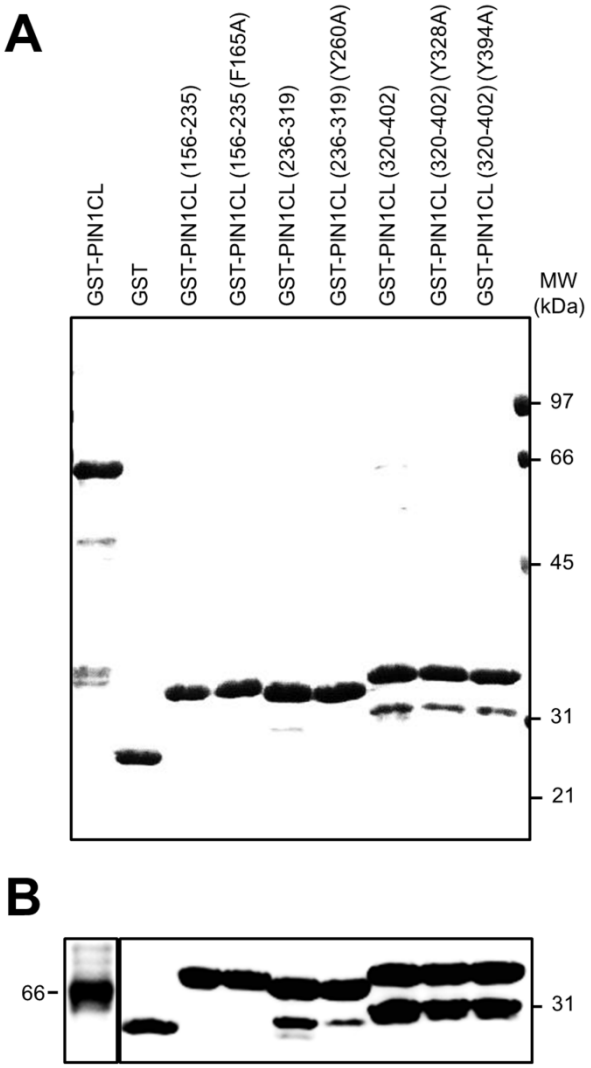
Supplemental Figure S1. **Alignment of the cytosolic loop of PIN proteins.**
Alignment of the amino acid sequence of the cytosolic loop of PIN1 with the sequences of the other members of PIN family (constructed using ClustalW). The four residues that were mutated to alanine in the PIN1-GFP mutant versions are indicated by asteriscs.

Supplemental Figure S2



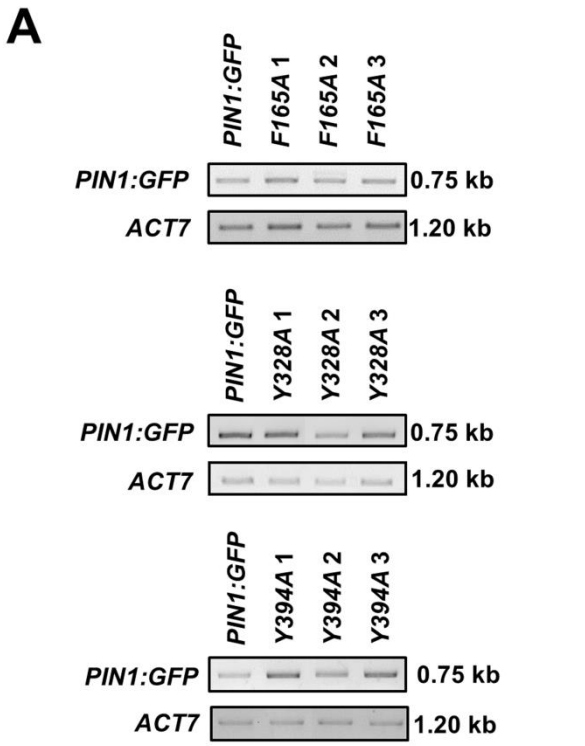
Supplemental Figure S2. **Purification of His-RBD-μ adaptins.** Bacterial extracts were applied to a His-trap column and bound proteins were eluted in the presence of increasing concentration of imidazole and analised by SDS-PAGE and Coomasie blue staining (upper panel) or Western blotting with histidine antibodies (WB). μ-adaptins were mainly collected at fraction 9 (F9), upon elution with 250 mM imidazole.

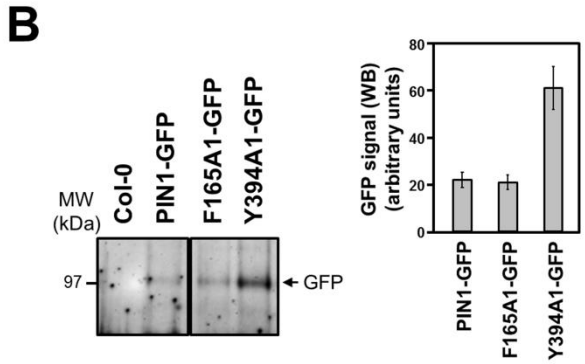
Supplemental Figure S3



Supplemental Figure S3. **Purification of GST-PIN1CL and GST-PIN1CL portions.** Bacterial extracts that express the complete cytosolic loop of PIN1 (residues156-482) or different regions of this loop (residues156-235, 236-319 or 320-402) and the corresponding mutant versions were affinity purified with glutathione-sepharose beads and analysed by SDS-PAGE and Coomassie blue staining (A) or Western blot analysis with GST antibodies (B).

Supplemental Figure S4



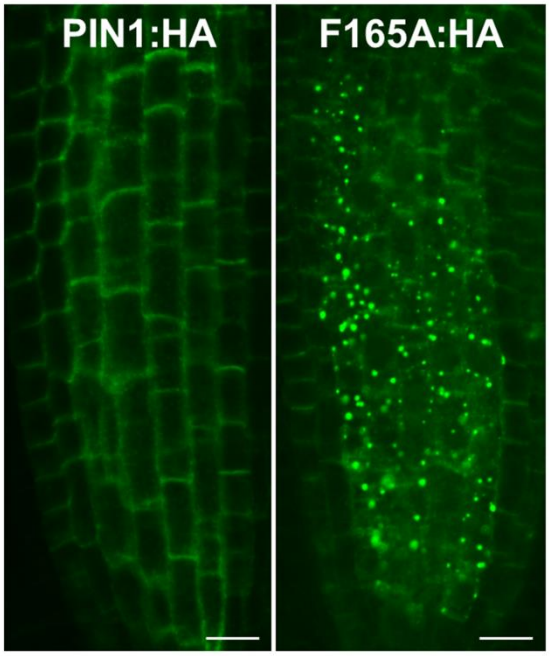


C

PIN1:GFP mutant lines	*pin1* phenotype plants
Y260A 1	20
Y260A 2	19
Y260A 3	33
Y328A 1	27
Y328A 2	91
Y328A 3	18
Y394A 1	23
Y394A 2	85
Y394A 3	24

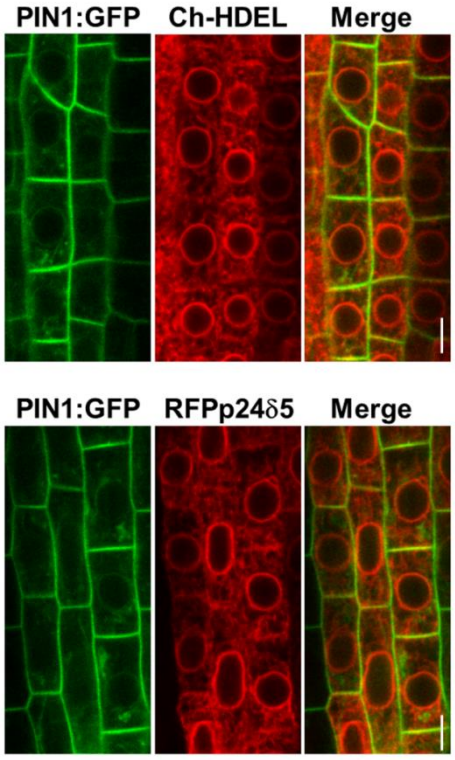
Supplemental Figure S4. **RT-PCR, Western Blot and phenotypic analysis in *pin1* background of PIN1:GFP-F165A, PIN1:GFP-Y260A, PIN1:GFP-Y328A and PIN1:GFP-Y394A lines. A.** Total RNA from seedlings of PIN1:GFP and lines 1-3 of every mutant were used for the RT-PCR. In the PCRs, specific primers of PIN1:GFP (LPM pin and GFP3 primers) were used. Actin-7 (ACT7) (A3 and A5 primers) was used as a control. PCR samples were collected at cycle 22 for ACT7 and at cycle 32 for PIN1:GFP lines.See text for details. Out of the three lines of PIN1:GFP-F165A analysed, one of them did not rescue the *pin1* phenotype (line2) and the other two did it partially (lines 1 and 3)(see Figure 3). Lines 1 and 3 of PIN1:GFP-Y328A showed no defects; in contrast, line 2 of PIN1:GFP-Y328A showed not rescue of the *pin1* phenotype probably due to PIN1:GFP-Y328A low levels of expression (see phenotypic analysis in C). Line 2 of PIN1:GFP-Y394A showed not rescue of the *pin1* phenotype; in contrast, lines 1 and 3 of PIN1:GFP-Y394A showed no defects probably due to PIN1:GFP-Y394A high levels of expression in these lines. **B**. Protein extracts from seedlings of Col-0, PIN1-GFP, F165A1-GFP and Y394A1-GFP lines were analysed by Western blotting with GFP antibodies. Right panel shows the quantification of the GFP signal of these lines by Western blotting. n = 3 independent experiments. Error bars represent standard deviation. **C**. Table shows the phenotypic analysis of 350 F2 plants from the cross of *pin1* and three independent homozygous lines of PIN1:GFP-Y260A (Y260A 1-3), PIN1:GFP-Y328A (Y328A 1-3) and PIN1:GFP-Y394A (Y394A 1-3) (analysis of PIN1:GFP-F165A is shown in Figure 3).

Supplemental Figure S5



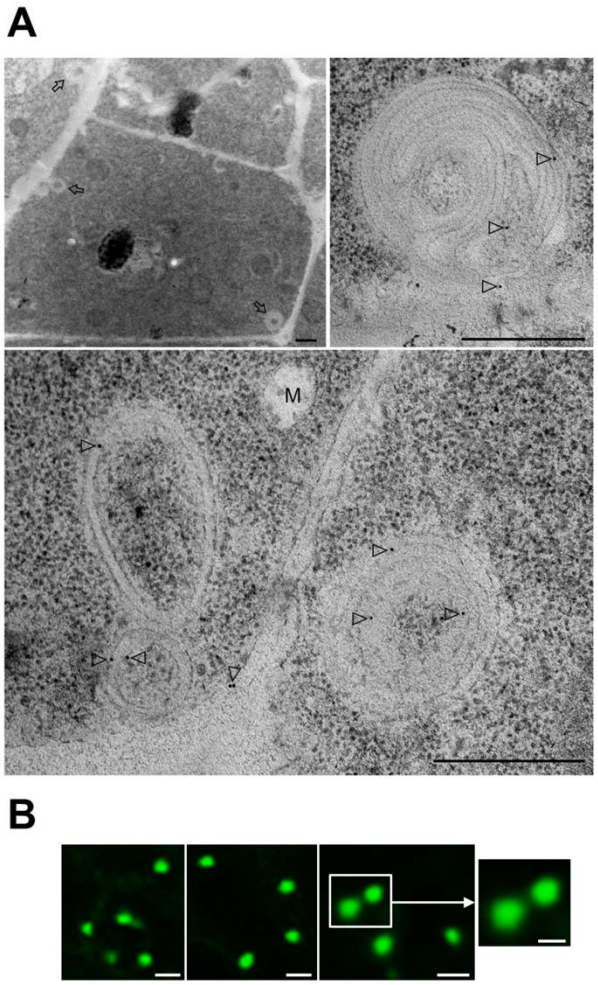
Supplemental Figure S5. **Localization of PIN1:HA and PIN1:HA-F165A mutant in *A. thaliana* roots.** Immunolocalization of primary roots of 4-days-old seedlings expressing PIN1:HA and the PIN1:HA-F165A mutant using anti-HA antibodies. Scale bar: 10 μm

Supplemental Figure S6



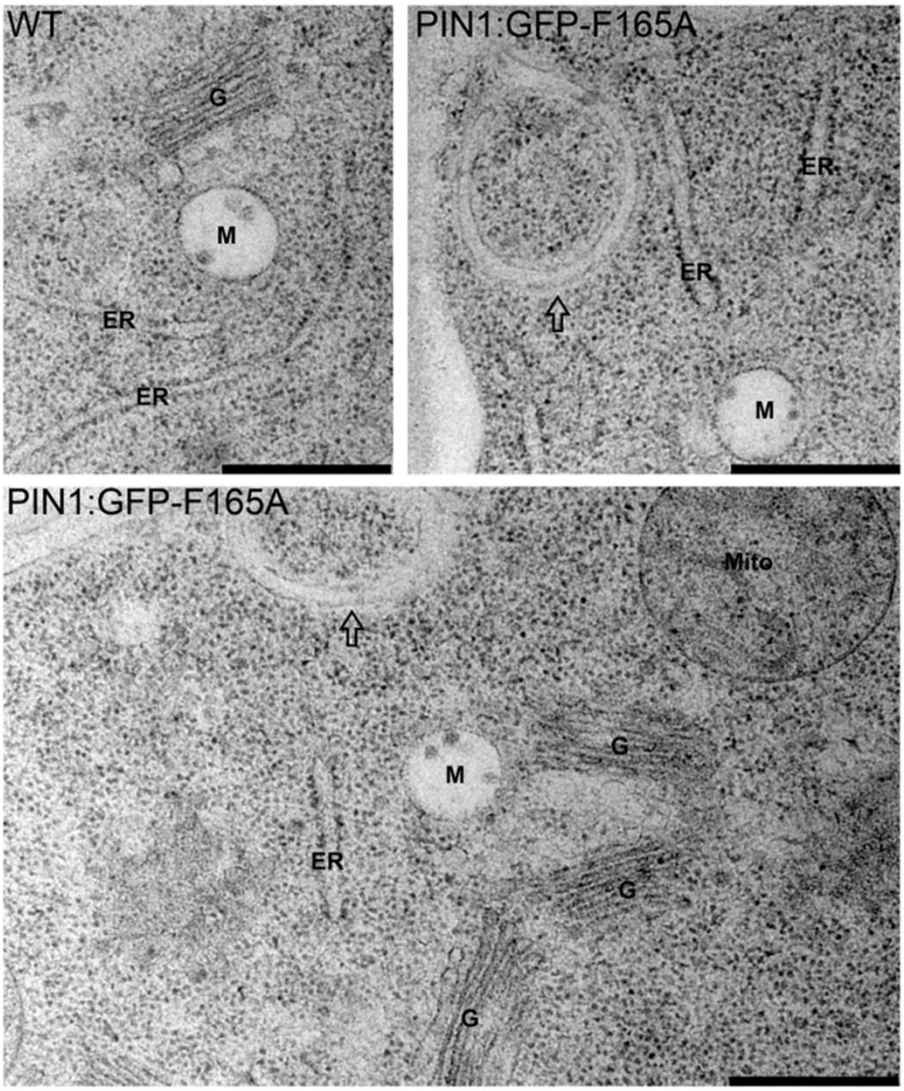
Supplemental Figure S6. Localization of mCherry-HDEL and RFP-p24δ5 in PIN1:GFP plants. CLSM of primary roots of 4-days-old seedlings expressing PIN1:GFP and two different ER markers, mCherry-HDEL and RFP-p24δ5. Scale bar: 10 μm.

Supplemental Figure S7



Supplemental Figure S7. Localization of PIN1:GFP-F165A by immunogold labeling (A) and CLSM (B) in roots of seedlings expressing PIN1:GFP-F165A.
A. Labelling with GFP antibodies showed that the PIN1:GFP-F165A mutant accumulates in big structures, from 200 to 500 nm of diameter, in stele cells. Lower magnification (left upper panel) shows that these structures (labelled by arrows) are often localized near the PM. A higher magnification shows that these structures contain multiple membranes. Arrowheads point to gold particles. Scale bars: 500 nm.
B. CLSM of primary roots of 4-days-old seedlings expressing the PIN1:GFP-F165A mutant. Scale bars: 1 μm (Inset: 500 nm).

Supplemental Figure S8



Supplemental Figure S8. Ultrastructural analysis of the PIN1:GFP-F165A mutant. Arrows point to the multimembrane structures found in the PIN1:GFP-F165A mutant but not in the wild-type (WT). ER, endoplasmic reticulum; G, Golgi apparatus; M, multivesicular bodies; Mito, mitochondria. Scale bars: 500 nm.

Supplemental Table S1. Primers used for PCR amplification.

Name	Gene	Sequence (5'→3')
Act3	*Actin-7*	GGAAAACTCACCACCACGAACCAG
Act5	*Actin-7*	GGATCCAAATGGCCGATGGTGAGG
GFP3	*GFP*	GGATCCATCCCAGCAGCTGTTACAAACTC
LBb1	T-DNA	GGATCCGCGTGGACCGCTTGCTGCAACT
LPIN	*PIN1*	CAAAAACACCCCCAAAATTTC
LPM pin	*PIN1*	CTCTGTACCTCAGGGGAATAG
RPIN	*PIN1*	AATCATCACAGCCACTGATCC

Supplemental Table S2. Primary antibodies used for Western blotting and immunolocalization.

Antibody	Host	Dilution	Reference
Western blotting			
μ2-adaptin	Mouse	1:100	BD Biosciences
Clathrin heavy chain	Mouse	1:1000	BD Biosciences
Histidine	Mouse	1:3000	GE Healthcare
GFP	Rabbit	1:500	Invitrogen
Immunolocalization			
GFP	Mouse	1:500	Sigma Aldrich
GFP	Rabbit	1:500	Invitrogen
HA	Mouse	1:500	Abcam
BiP	Mouse	1:200	Assaydesigns
Sar1	Rabbit	1:800	Yang et al., 2005
ARA7	Rabbit	1:100	Haas et al., 2007
Sec21	Rabbit	1:800	Movafeghi et al., 1999
MIN7	Rabbit	1:1000	Nomura et al., 2006
E2	Rabbit	1:250	Wang et al., 2010
SH3-P2	Rabbit	1:200	Zhuang et al., 2014
Anti-Rabbit IgG 10 nm	Goat	1:50	Electron Microscopy Sciences

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