Supplemental Table.

Table S1. Fluorophore characteristics of used fluorescent proteins, Related to Figure 2 and 3.

Transgenic line	Fluprescent marker	Aminoacid change in chromophore	pK(a)	Ref.
PIN3::PIN3-GFP	GFP	S65 (no change)	(5.9)	(Kneen et al., 1998)
PIN1::PIN1:GFP-1	mGFP4	S65 (no change)	(5.9)	(Haseloff and Amos, 1995)
PIN7::PIN7:GFP	mGFP4	S65 (no change)	(5.9)	(Haseloff and Amos, 1995)
PIN2::PIN1-GFP2, PIN2::PIN1-GFP3	EGFP	F64L, S65T, H231L	**(**6.16)	(Llopis et al., 1998)
SKU5::SKU5-GFP	EGFP	F64L, S65T, H231L	**(**6.16)	(Llopis et al., 1998)
PIN2::PIN2-GFP	EGFP	F64L, S65T, H231L	**(**6.16)	(Llopis et al., 1998)
AUX1::AUX1-YFP N-terminal, AUX1::AUX1-YFP 116	EYFP	S65G,S72A,T203Y, H231L	(7.1)	(Llopis et al., 1998)
PIN1::PIN1-YFP	EYFP*	S65G,V68L,Q69M,S72A,T203Y	(5.7)	(Griesbeck et al., 2001)
SYP22::SYP22-YFP	EYFP*	S65G,V68L,Q69M,S72A,T203Y	(5.7)	(Griesbeck et al., 2001)

*EYFP version described in Griesbeck et al., 2001 is a version derived from the one published by Llopis et al., 1998 but with additional fluorophore stability enhancing mutations. Both versions were named EYFP in literature to make the distinction we used the asterisk.

Supplemental Figures.

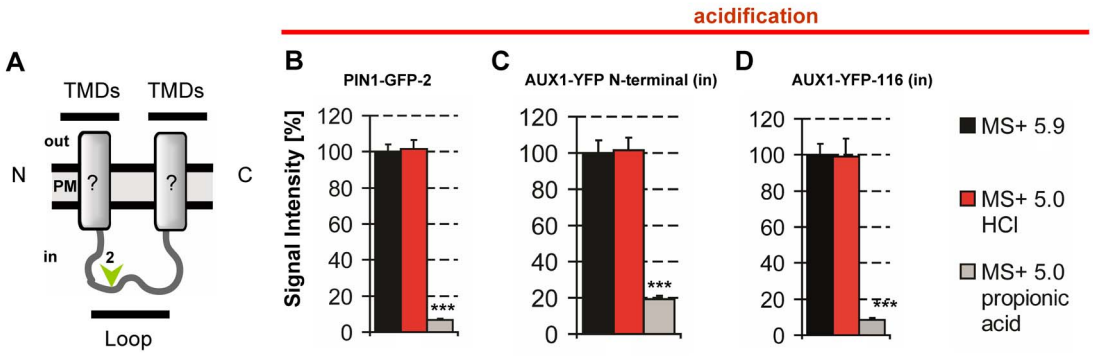


Figure S1. Distinct acidification treatments of various plasma membrane proteins tagged with GFP or YFP fluorescent proteins, Related to Figure 2.
(A) The presumptive simplified 2D model of PIN1 protein where the GFP insertion positions are indicated by green arrow heads (for the exact position of GFP insertions see Figure 1A).
(B - C) Quantification of fluorescence signal changes recorded for reporter lines PIN1-GFP-2 **(B)**, AUX1-YFP N-terminally tagged with the reporter moiety positioned in the cytoplasm (in) **(C)** and AUX1-YFP-116 (in) **(D)** after incubation with media acidified to pH 5.0 with either membrane non-permeable hydrochloric or membrane permeable propionic acid in comparison to the mock treatment (control media buffered with KOH to pH 5.9). Quantification of the fluorescent signal changes recorded for the respective reporter lines after 30 min treatment; mock treatment is plotted as 100%, error bars represent SE for 3 biological repeats (number of seedlings imaged, n > 7 per each replicate; asterisk represent P-values: *** - P < 0,001).

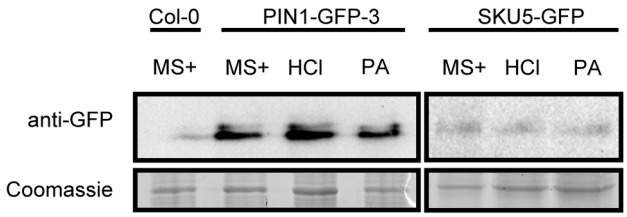


Figure S2. PIN1-GFP-3 and SKU5-GFP do not undergo proteolytic degradation after acid treatment, Related to Figure 2 and 3.

Western blot analysis of PIN1-GFP-3 (left hand side) and SKU5-GFP (right hand side) proteins isolated from seedlings subjected to, analogical as in the fluorescence quenching measurements, 30 min HCl or propionic acid (PA) treatments. Proteins were detected using an antibody against the GFP moiety (upper panels). Commassie gel loading control is visible on the bottom panels. Wild-type Columbia (Col-0) ecotype was used as no GFP control.

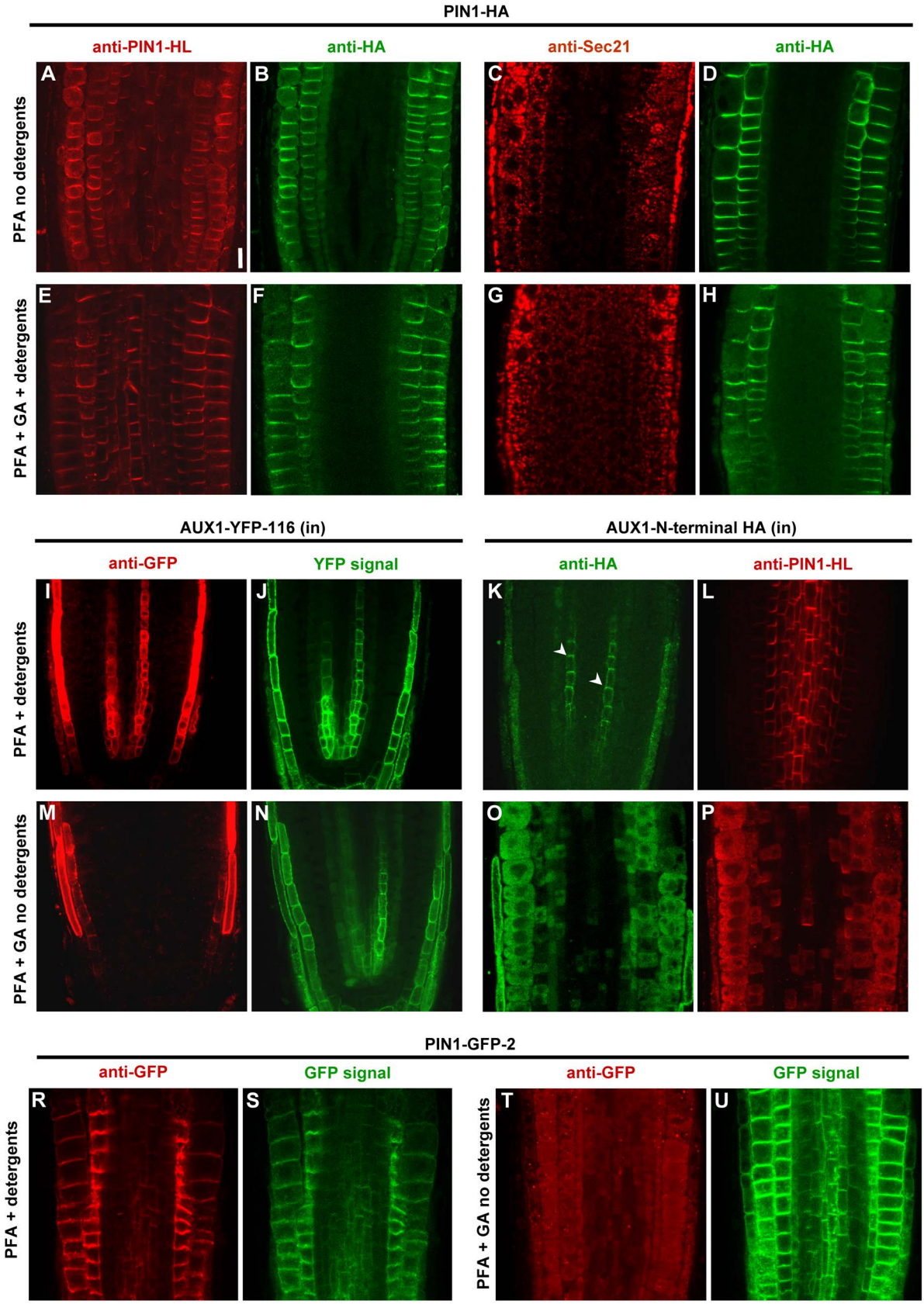


Figure S3. Immunolocalization protocols where paraformaldehyde fixation is used without detergents or paraformaldehyde and glutaraldehyde fixation is used with detergents both result in membrane permeability, Related to Figure 4.

(A-D) Antibody staining of epitopes expressed in root cells of *Arabidopsis* PIN1-HA transgenic line after paraformaldehyde (PFA) fixation alone, without lipid fixation and detergents, results in labelling of the PIN1 hydrophilic loop (PIN1-HL**; A**) and the cytoplasmatically located Sec21 (**C**; red) as well as with a prominent HA tag signal present (**B**, **D**; green).
(E-H) Immunolocalization after application of the lipid fixing paraformaldehyde (PFA) and glutaraldehyde (GA) mixture but with addition of detergents results in more unified tissue labelling for all the epitopes concerned similar to the standard membrane permeable immunolocalization protocol.
(I-L) Immunolocalization protocol, performed on *Arabidopsis* roots, utilizing paraformaldehyde (PFA) fixation only without detergent permeabilization results in labelling of the cytoplasmatically located YFP with anti-GFP antibody (red; **I**) with the fluorescent signal of YFP alone also visible (green; **J**) in AUX1-YFP-116 transgenic line; similarly anti-HA (green, **K**) and anti-PIN1-HL (red; **L**) antibody signals are visible in the HA-AUX1 expressing roots where the tag is fussed N-terminally to the AUX1 protein and positioned in the cytoplasm.
(M-P) Immunolocalization after fixation with paraformaldehyde (PFA) and glutaraldehyde (GA) mixture, without detergents used in the protocol, results in visibly weaker labelling of cytoplasmatically localized epitopes targeted by anti-GFP **(M)**, anti-HA **(O)**, anti-PIN1-HL antibodies respectively with the fluorescent signal of YFP alone also visible **(N)**.
(R-U) Anti-GFP Antibody staining of PIN1-GFP-2 in membrane permeable conditions with addition of detergents and paraformaldehyde (PFA) fixation results in a pronounced labelling of the GFP (red, **R**) with the fluorescence of the reporter itself is also visible (green, **S).**
Immunolocalization in membrane non-permeable conditions without detergents and with prior fixation by PFA and glutaraldehyde (GA) mixture results in absence of the red signal corresponding to the labelling of the GFP by antibodies **(T)** while the green signal of the reporter itself remains visible **(U)**. Bars = 10 μm.

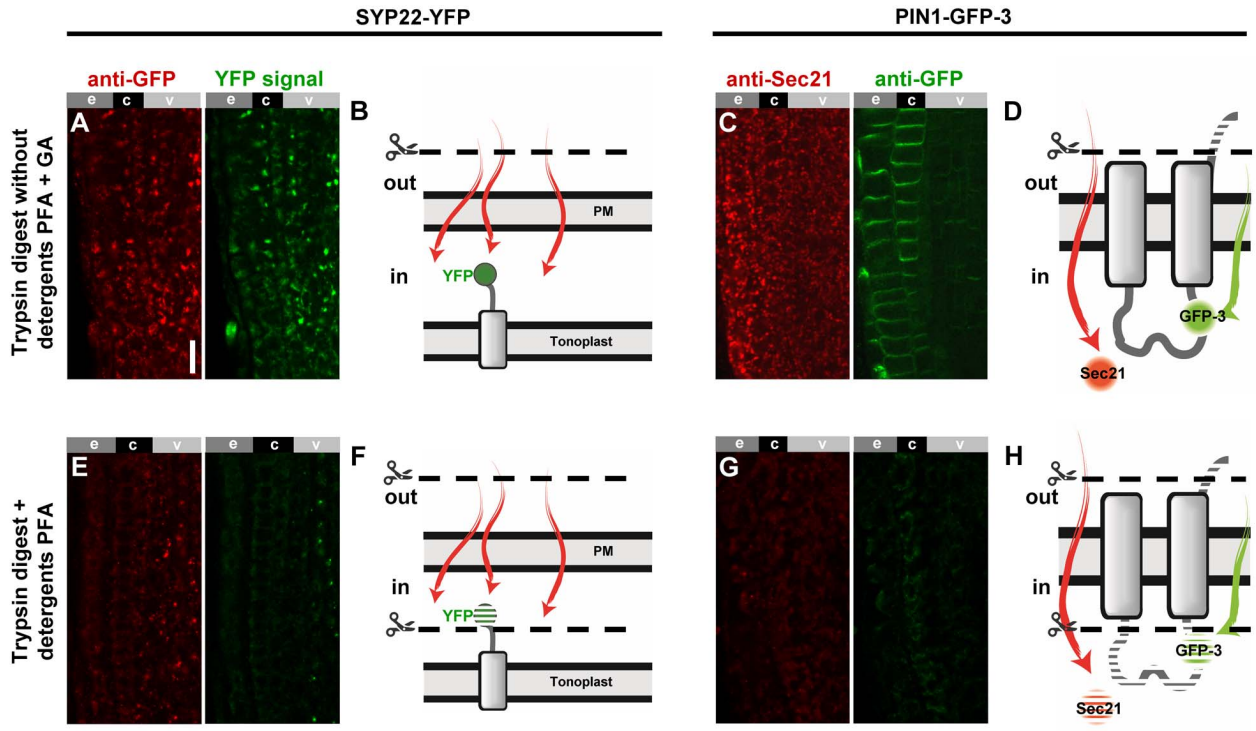


Figure S4. Limited membrane permeability prevents tryptic digestion of intracellular markers SYP22-YFP, the Golgi marker Sec21 and intracellular epitopes of PIN1-GFP-3 but enables antibody permeability, Related to Figure 5.

(A, C, E, G) Antibody staining and the native green YFP signal of SYP22-YFP **(A, E)**, immunolabelling of Sec21 (red) and PIN1-GFP-3 (green; **C, G**) after tryptic digest in membrane non-permeable **(A, C)**, versus membrane permeable conditions **(E, G)**. Bars = 10 µm.

(B, D, F, H) Interpretative cartoons depicting the intracellular position of SYP22-YFP (**B, F**; green), Sec21 (**D, H;** red) and the presumptive orientation of PIN1 hydrophilic loop with the GFP-3 moiety (**D, H;** green). Arrows indicate the accessibility of respective epitopes to anti-GFP (red, **A, E** left hand side panels), anti-Sec21 (red; **C, G** left hand side panels) and anti-GFP (green; **A, E, C, G** right hand side panels) antibodies. Shaded areas reflect destruction of the epitopes after tryptic digest in membrane non-permeable **(B, D)** vs. permeable **(F, H)** conditions. The designations (in) and (out) refer to the intracellular and the extracellular space respectively. Bars = 10 µm.

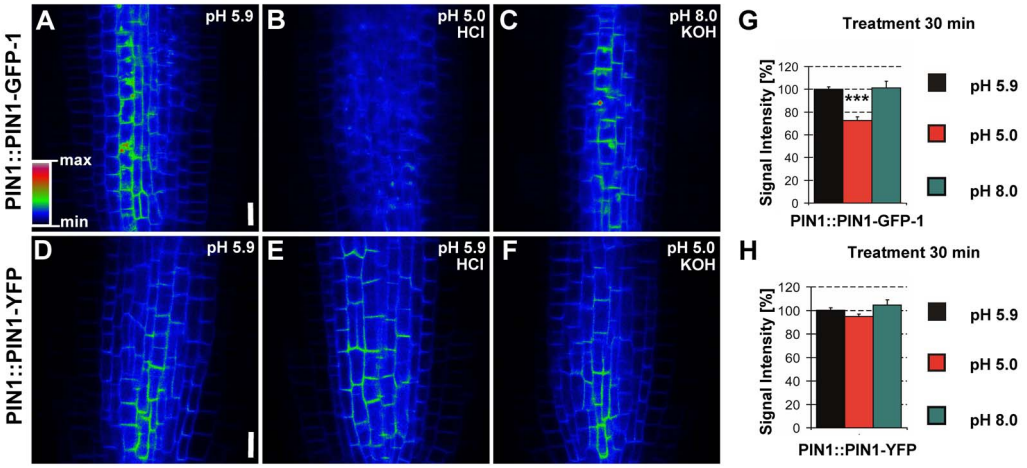
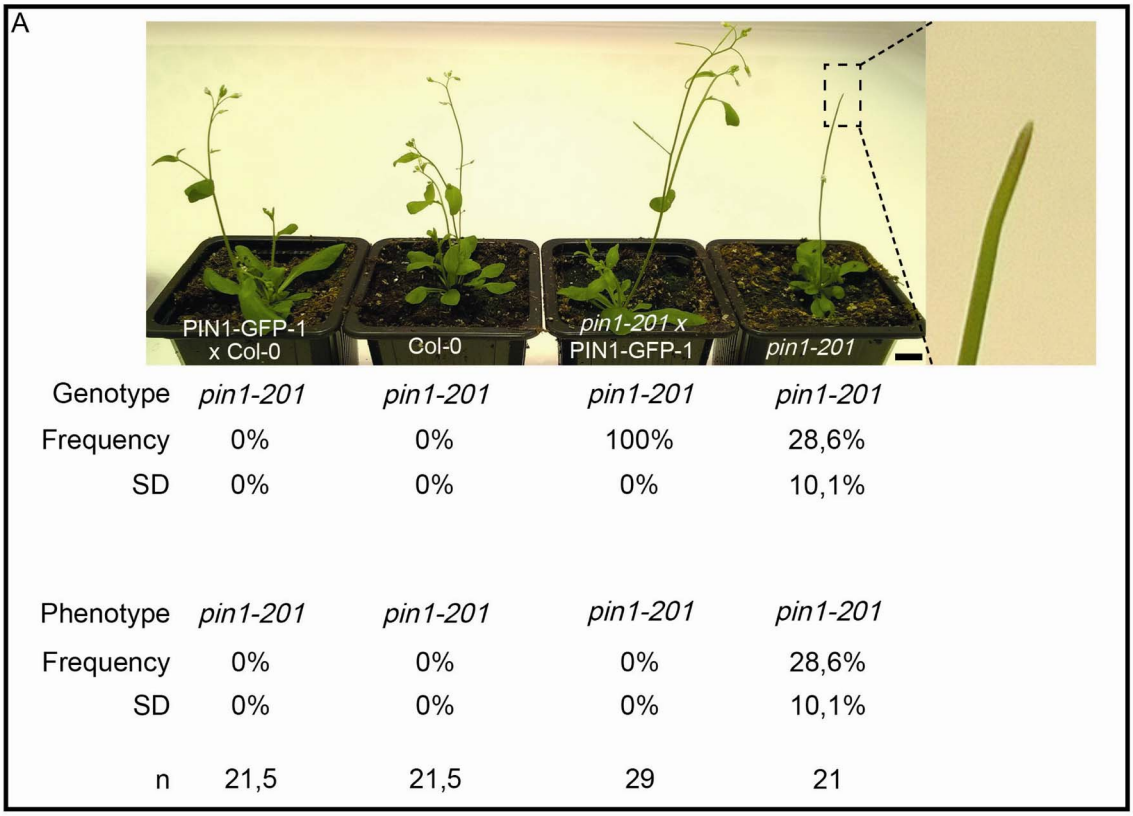


Figure S5. Fluorescence of the GFP-1 reporter of PIN1 intragenic fusion is affected decreased by acidification of the extracellular environment, Related to Figure 6.

(A - C) Exchange of the control medium pH 5.9 by medium acidified to pH 5.0 with membrane non-permeable hydrochloric acid (HCl) reduces the fluorescence of PIN1::PIN1-GFP-1 **(B)** in comparison to the control **(A)** while analogous treatment with pH 8.0 KOH titrated medium does not affect the PIN1-GFP-1 signal **(C)**.

(D - F) Analogous treatments do not visibly affect the PIN1::PIN1-YFP fluorescence. Fluorescence intensities are colour-coded.

(G, H) Quantification of fluorescence signal changes recorded for PIN1::PIN1-GFP-1 **(G)** and PIN1::PIN1-YFP **(H)** after 30 minutes treatment with media titrated to pH 5.0 or pH 8.0; control treatment is plotted as 100%, error bars represent SE for 3 biological repeats (number of seedlings imaged, n > 10 per each replicate; asterisk represent P-values: *** - P < 0,001). Bars = 10 µm.



Genotype	*pin1-201*	*pin1-201*	*pin1-201*	*pin1-201*
Frequency	0%	0%	100%	28,6%
SD	0%	0%	0%	10,1%
Phenotype	*pin1-201*	*pin1-201*	*pin1-201*	*pin1-201*
Frequency	0%	0%	0%	28,6%
SD	0%	0%	0%	10,1%
n	21,5	21,5	29	21

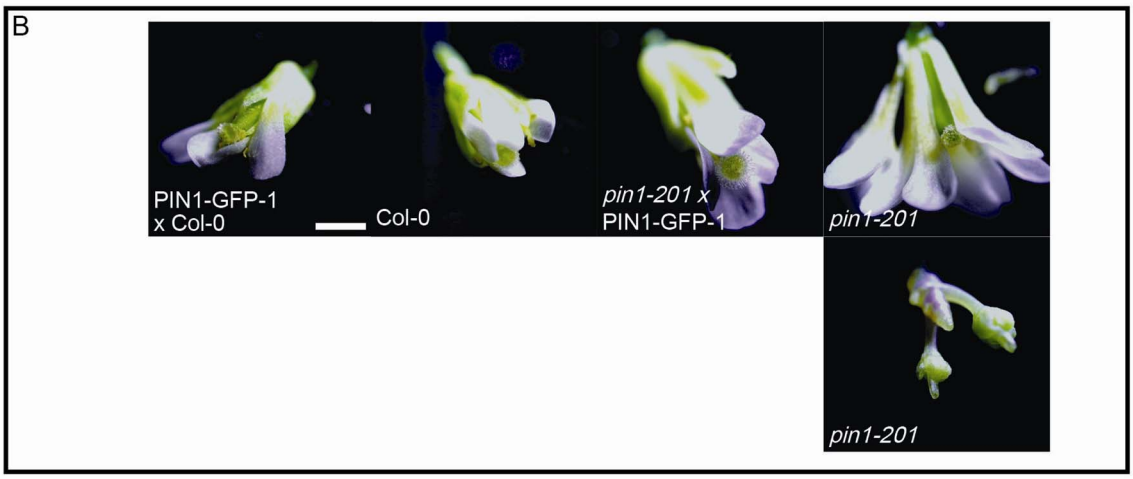


Figure S6. PIN1-GFP-1 construct complements the sterile *pin1-201* null allele, Related to Figure 6.
(A) Grown plant phenotypes of 4-week-old plants. Left to right: PIN1-GFP-1 in Columbia background (PIN1-GFP-1 x Col-0), Columbia wild-type control (Col-0), double homozygous cross of PIN1-GFP-1 with *pin1-201* (*pin1-201* x PIN1-GFP-1) and the homozygous *pin1-201* plant showing the characteristic pin-like inflorescence magnified in the insert on the right; the frequencies (%) of *pin1-201* genotype and phenotype (pin-like inflorescence) for plant lines mentioned above were calculated after evaluating an average number of (n) plants indicated, SD represents the standard deviation for 2 biological repeats, bar = 1 cm.
(B) Flower phenotypes of 4-week-old plants, plant line pictures arranged left to right in a corresponding order to **(A)**, increased petal number, absence of stamens and gynoecium developmental defects observed only in *pin1-201* are depicted on the right hand side; bar = 1 mm.

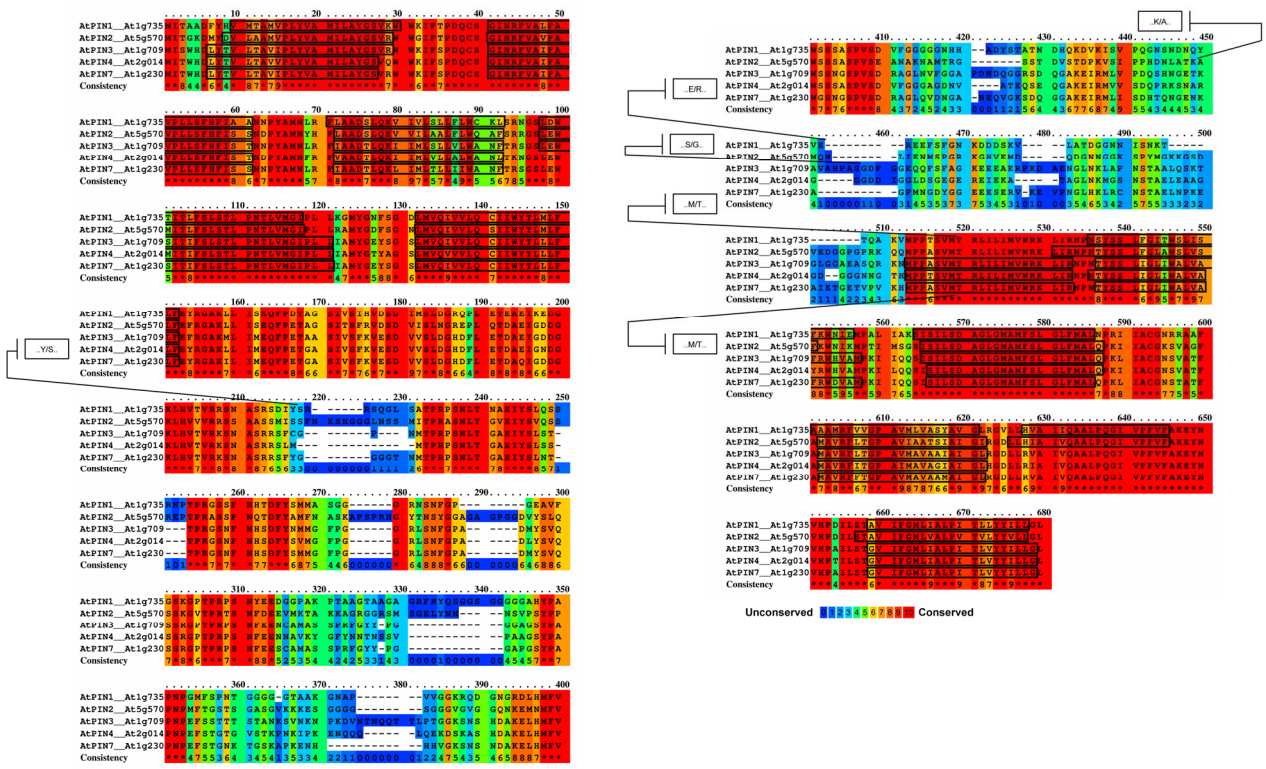


Figure S7. The amino acids constituting the transmembrane domains are conserved between *Arabidopsis* plasma membrane PINs, Related to Figure 7.
The depicted above alignment illustrates conservation by colouring the amino acids with warm colours reflecting a high degree of moiety conservation and cold colouring in instances of low conservation, additionally conservation is scored form highest 10 to lowest 0 values (see colour code legend at figure bottom) while full conservation is indicated by asterisks. The amino acids building the transmembrane domains are conserved between *Arabidopsis* plasma membrane PINs while the large hydrophilic loop region is much more divergent. The positions of GFP insertions in particular PINs are indicated by extension where the slash (/) signifies the GFP position between particular amino acids. The predicted transmembrane domains are enclosed in rectangles.

Supplemental references.

Griesbeck, O., Baird, G.S., Campbell, R.E., Zacharias, D.A. and Tsien, R.Y. (2001). Reducing the Environmental Sensitivity of Yellow Fluorescent Protein MECHANISM AND APPLICATIONS. J. Biol. Chem. 276:29188–29194.

Haseloff, J. and Amos, B. (1995). GFP in plants. Trends Genet. 11:328–329.

Kneen, M., Farinas, J., Li, Y. and Verkman, A.S. (1998). Green Fluorescent Protein as a Noninvasive Intracellular pH Indicator. Biophys. J. 74:1591–1599.

Llopis, J., McCaffery, J.M., Miyawaki, A., Farquhar, M.G. and Tsien, R.Y. (1998). Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. Proc. Natl. Acad. Sci. 95:6803–6808.