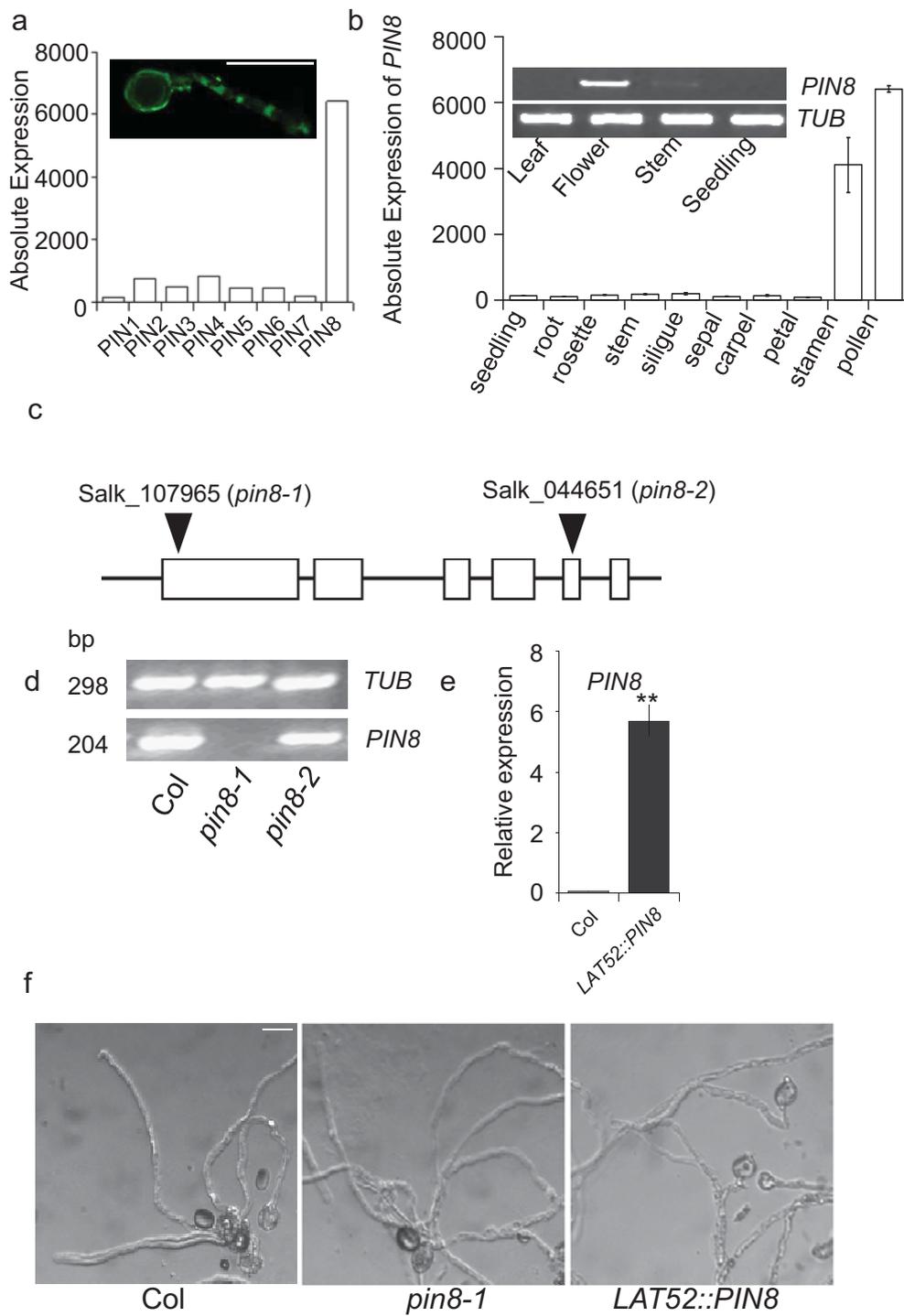


## **Supplementary Information**

### **ER-localized auxin transporter PIN8 regulates auxin homeostasis and male gametophyte development in *Arabidopsis***

## Supplemental Figures



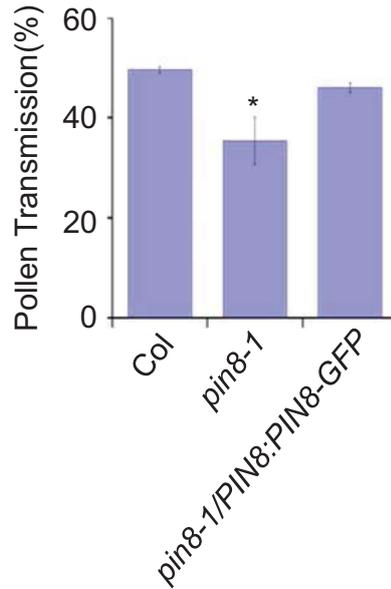
**Supplemental Figure S1| *PIN8* expression pattern and loss or ectopic expression lines.**

(a) Expression analysis of PIN proteins in pollen according to the publicly available microarray data (<https://www.genevestigator.ethz.ch/at/>), the insert shows that *PIN8* is expressed in pollens and pollen tubes in the *PIN8::PIN8-GFP* line.

(b) The expression analysis of *PIN8* in different tissues according to the public available microarray data (<https://www.genevestigator.ethz.ch/at/>). The insert was the confirmation of the microarray data by RT-PCR analysis. Tubulin was used as a control. (c) Structure of the *PIN8* gene

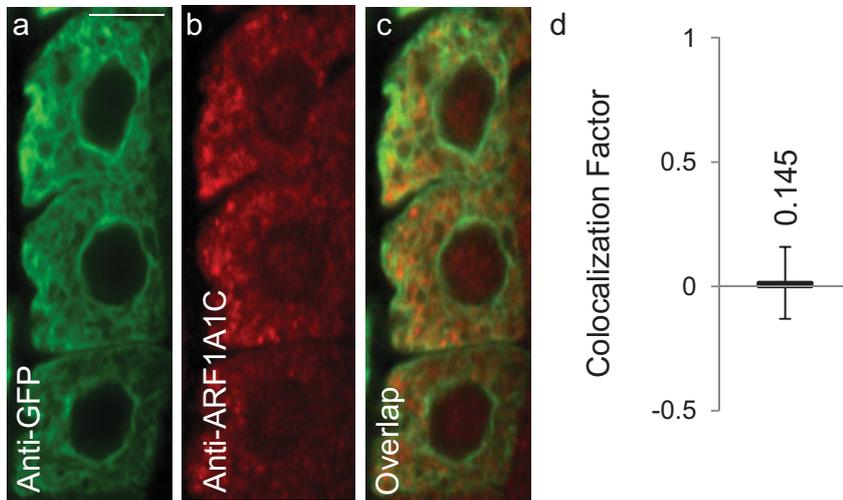
with six exons and depiction of two T-DNA insertions characterizing as *pin8-1* and *pin8-2*. (d) *pin8-1* is knock out allele which was confirmed by RT-PCR analysis. RNA for RT-PCR analysis

was isolated from flowers. Tubulin was used as a control. (e) The *LAT52::PIN8* line had highly increased *PIN8* transcription level compared to the wild type control. RNA for Q-PCR analysis was isolated from flowers. Tubulin was used as a relative control. Error bars represent standard error from the mean of three independent biological repeats (Student's *T* test, \*\**P* < 0.01). (f) Pollen germination was normal in both *pin8-1* and the *LAT52::PIN8* line. Bar = 25 μm.



**Supplemental Figure S2|PIN8::PIN8-GFP is functional.**

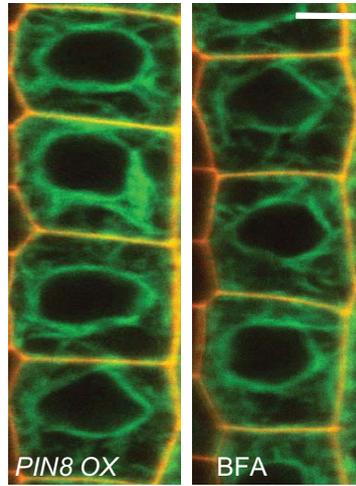
The functional *PIN8::PIN8-GFP* was confirmed via rescuing *pin8-1* loss function mutant pollen transmission ability, Error bars represent the standard error of more than 10 independent crosses (Student *T* test, \* $P < 0.05$ ).



**Supplemental Figure S3|PIN8 subcellular localization.**

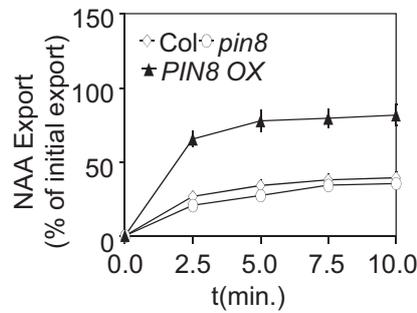
(a-c) PIN8 (anti-GFP, green) is not colocalized with endosome (anti-ARF1, red) in the *35S::PIN8-GFP* line. Bar = 5  $\mu$ m.

(d) The colocalization factor (the highest is 1.0) was calculated by Zeiss software. For microscope observation, 3-day-old seedlings ( $n=6$ ) and at least 5 areas were analyzed for each seedling. Error bars represent the standard error.



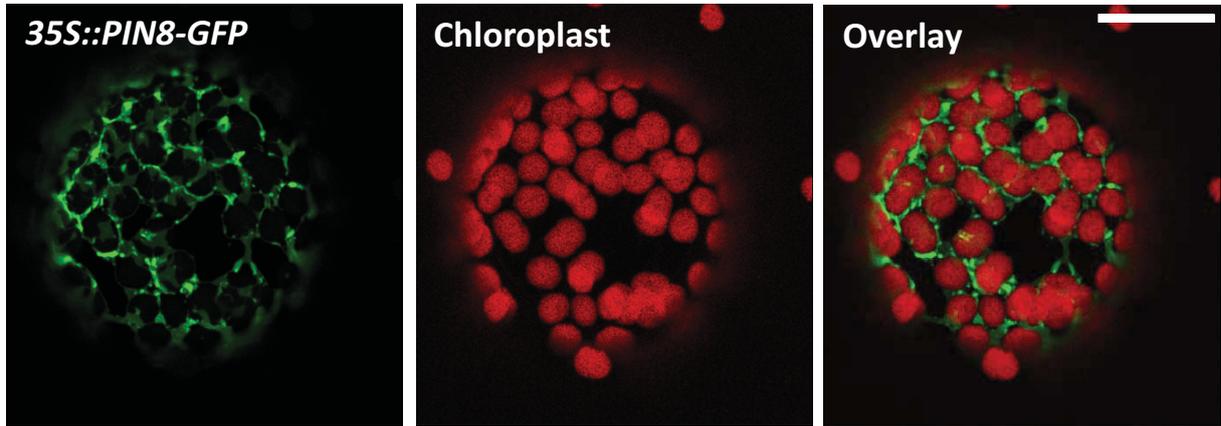
**Supplemental Figure S4| PIN8 localization is insensitive to trafficking inhibitor brefeldin A.**

The *35S::PIN8-GFP* seedlings were treated with brefeldin A (BFA, 50 $\mu$ M) for 2 hours before observation. Bar=5  $\mu$ m



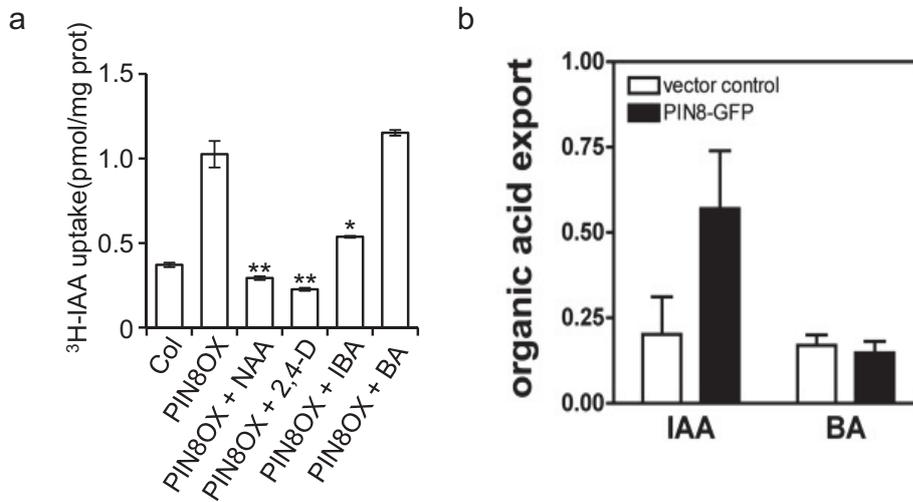
**Supplemental Figure S5| Protoplast transport assays.**

Protoplasts of PIN8OX showed a higher rate of NAA export. Error bars represent standard error of the mean, n = 3.



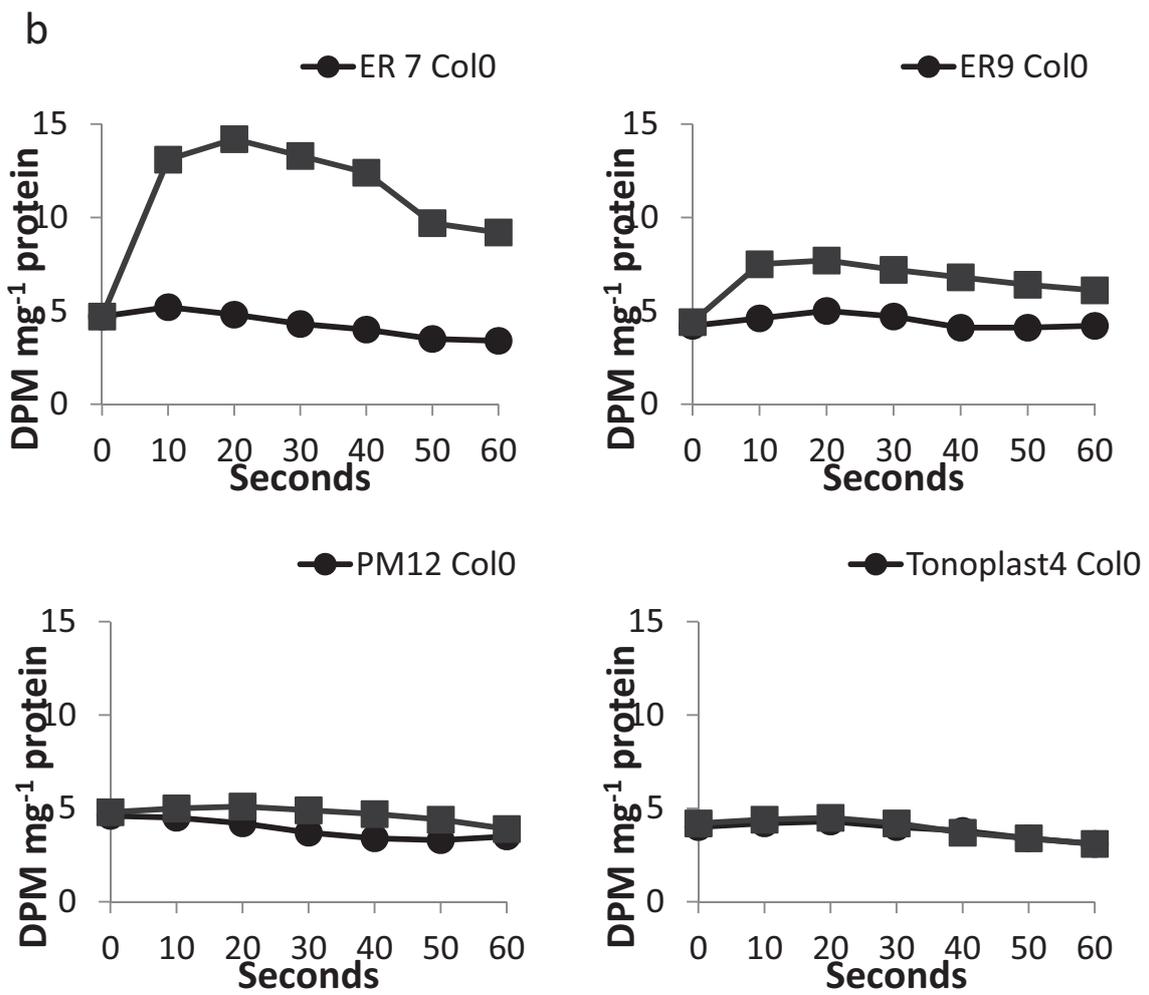
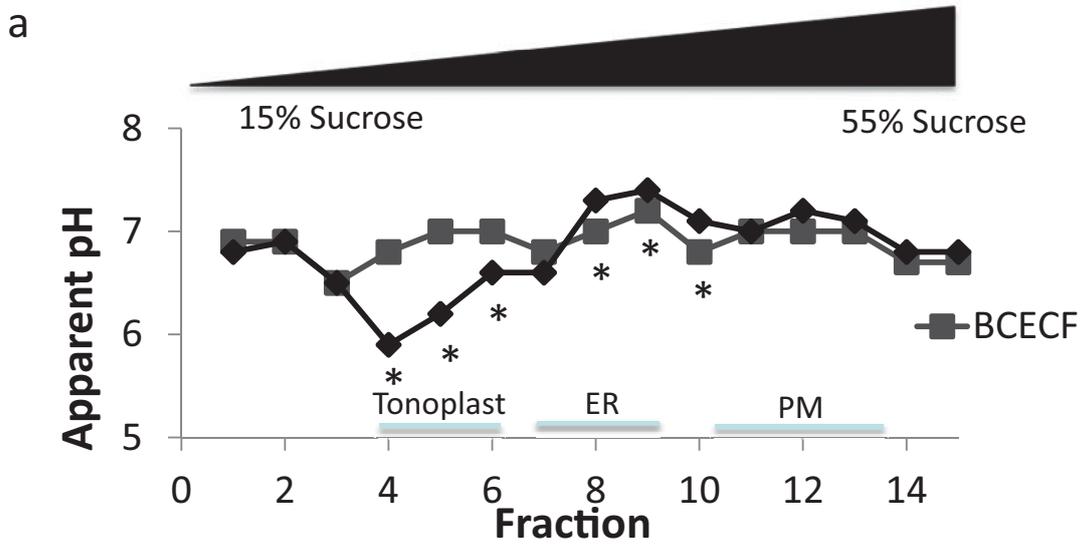
**Supplemental Figure S6| PIN8 is localized at ER in protoplasts from transiently transfected *Nicotiana benthamiana*.**

The red dots are chloroplasts, the bright green ring-like GFP signals corresponds to 35S::PIN8-GFP. Bar=10  $\mu$ m



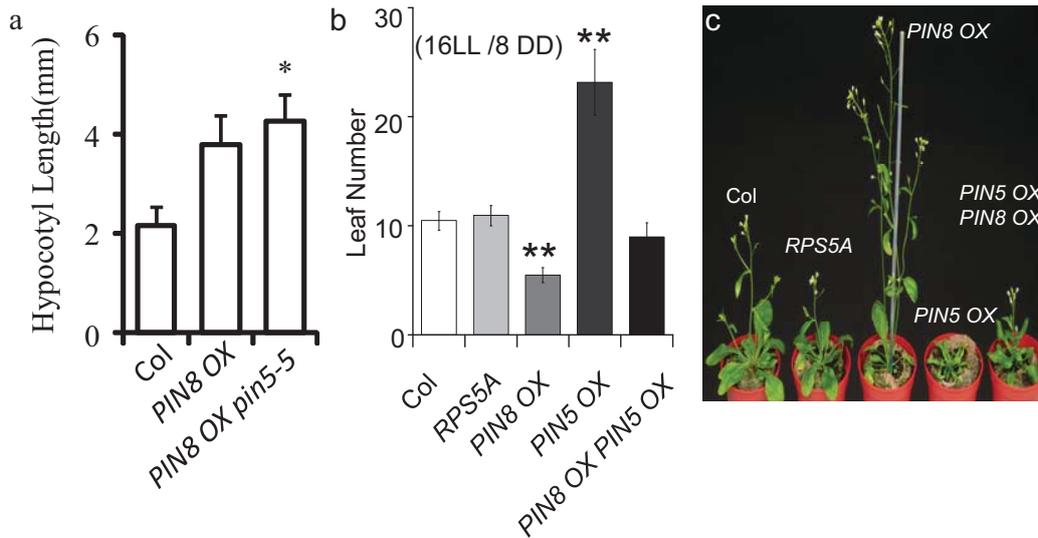
**Supplemental Figure S7| Biologically active auxins are preferred substrates of PIN8 action.**

- (a) The IAA uptake in vesicle fractions from *PIN8 OX* and the Col control was inhibited at the presence of the auxin analogous indole butyric acid (IBA, 1mM), NAA(1mM), 2,4-D(1mM), while the biologically inactive analogue benzoic acid (BA, 1mM) had no effect on the PIN8-mediated uptake of radiolabelled IAA. Error bars represent standard error of the mean, n = 3. (Student t test, \*P<0.05, \*\*P<0.05). Transport assays were performed as described in Material and Methods. ER-enriched membranes were incubated in 50 nM <sup>3</sup>H-IAA fractions. Aliquots were taken at 45" after start, filtered and washed. The radioactivity present in the filters was estimated by liquid scintillation counting and expressed as pmol of <sup>3</sup>H-IAA / mg of total proteins.
- (b) Auxin transport assays in mesophyll protoplasts from transiently transfected *Nicotiana benthamiana* indicated that IAA, not BA, was the preferred substrate of PIN8 action. IAA export analyses from *N. benthamiana* mesophyll protoplasts was analyzed 2-3 day agrobacterium-mediated co-transfection. Tobacco protoplast preparation and transport assays were identical to the described *Arabidopsis* protocol<sup>22</sup> except that a 25% percoll gradient was used. Relative IAA/NAA export is calculated from effluxed radioactivity as follows: ((radioactivity in the medium at time t) - (radioactivity in the medium at time t=0)) \* (100%) / (radioactivity in the medium at t=0). Presented are average values from 3 independent protoplast infiltrations.



**Supplemental Figure S8 | pH status and auxin transport in active, sealed membrane vesicles from *PIN8OX* seedlings.**

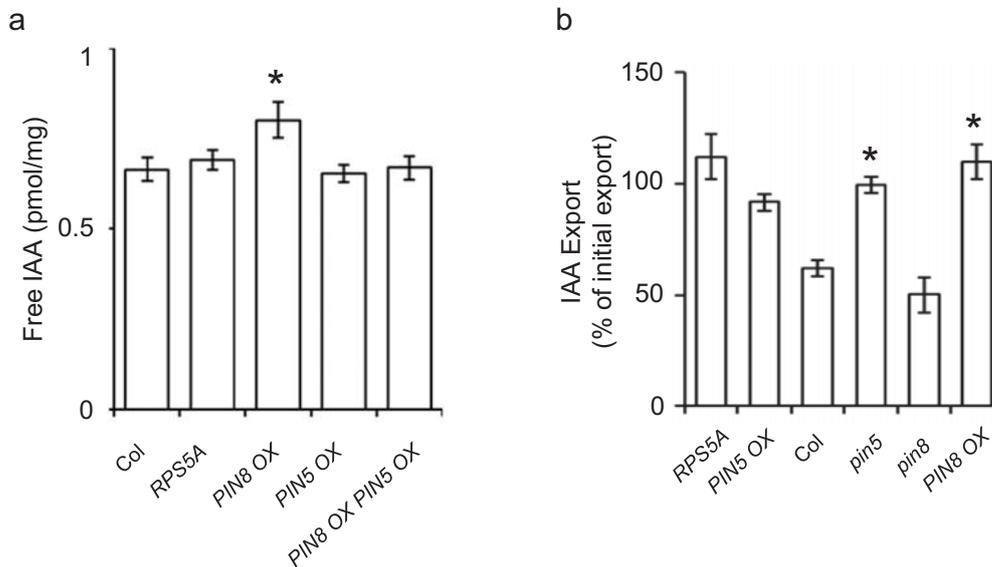
- a) Apparent pH determined from 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) fluorescence of washed vesicles incubated for 5 min  $\pm$  Mg-ATP. Tonoplast fractions (4-6) and, to a lesser extent, lighter ER fractions (7) exhibit acidification in the presence of ATP. More dense ER fractions (8, 9) exhibit slight ATP-induced alkalinization. Collapse of a  $H^+$  gradient by gramicidin resulted in neutral (buffer) pH readings. Readings below pH 6.5 were confirmed using Oregon Green. pH measurements BCECF (20  $\mu$ g/mL) was added to 100  $\mu$ L fractions which were then incubated on ice for 5 min, then centrifuged at 100,000 x g for 15 min. Pellets were washed with 500  $\mu$ L buffer, centrifuged at 25,000 x g for 5 min and were resuspended in 100  $\mu$ L for fluorometric assays (Synergy HT, Biotek, Burlington, VT). Gramicidin (10  $\mu$ M) was added to collapse gradients. Membrane fractions were analyzed by ELISA and/or western blotting with antisera for subcellular marker proteins AVP1, VHA1 for tonoplast, BIP, PDI for ER; AHA2, ABCB4 for PM). Values are means and SD, n=3. Asterisks indicate difference by Student's t- test ( $P < 0.05$ )
- b) ATP-treated lighter ER membranes (7) from lines overexpressing PIN8 accumulated  $^3H$ -IAA in short-term (60 s) uptake assays. Denser ER vesicles, tonoplast vesicles, and PM vesicles did not show a similar activity. Uptake was not inhibited by addition of 0.5 mM benzoic acid buffered with 5 mM BTP-MES (pH7.0). Assays were repeated twice with similar results. ER7, ER fraction 7; ER9, ER fraction 9; PM12, plasma membrane fraction 12; T4, tonoplast fraction 4. Total membranes from 6-day-old *Arabidopsis* seedlings were fractionated on a sucrose density gradient and assayed for pH and  $^3H$ -IAA uptake with and without addition of ATP.



**Supplemental Figure S9|PIN5OX and PIN8OX have antagonistic functions.**

(a) *pin5* enhanced long hypocotyls phenotype of *PIN8 OX*. Error bars represent standard error from the mean of 2 independent experiments (n=44, Student T test, \*\*P<0.05).

(b-c) The earlier flowering phenotype of the *PIN8OX* line was rescued in the *PIN8OX PIN5OX* line. Plants were grown under long-day conditions (16-hour light/8-hour dark).



**Supplemental Figure S10|PIN8 and PIN5 have antagonistic roles in both auxin transport and auxin homeostatic regulations.**

(a) The increased free IAA phenotype of the *PIN8OX* line was rescued in the *PIN8OX PIN5OX* line. Error bars represent standard error of the mean, n = 4 (Student T test, \*P<0.05).

(b) Protoplasts of *PIN8OX* showed a higher rate of IAA export compared to the Col control, similar to *pin5*. Error bars represent standard error of the mean, n = 3. (Student T test, \*P<0.05).

## Supplemental Tables

### Supplemental Table S1

	<i>pin 8-1</i>	<i>pin5-5</i>	Col
	% (st. dev.)	% (st. dev.)	% (st. dev.)
aborted pollen	13.96 (15.07)	10.92 (11.26)	4.44 (2.26)
binuclear pollen	11.3 (4.23)	10.63 (3.9)	7.56 (2.67)
eccentric nuclei	5.3 (2.14)	5.96 (3.54)	2.15 (2.37)
misshaped MGU	15.22 (4.77)	16.25 (5.45)	8.56 (3.65)
linear MGU	21.74 (3.79)	20.21 (5.3)	5.92 (3.78)
1-nuclear pollen	1.65 (2.03)	1.46 (2.08)	0.73 (1.41)
Wt-looking	63.48	63.12	85.58

### Supplemental Table S2

	Cell Length, $\mu\text{m}$
Col	85 $\pm$ 3
PIN8 OX	175 $\pm$ 5

Measurements of the length of epidermal cells in hypocotyls of 5-day old seedlings of *PIN8 OX* and Col. The data represent the mean of 50 measurements from 5 seedlings. The difference between Col and *PIN8 OX* is significantly different ( $P < 0.001$ ).

### Supplemental Table S3

*TUBF*: ACTCGTTGGGAGGAGGAACT

*TUBR*: ACACCAGACATAGTAGCAGAAATCAAG

*PIN8F*: TGGCCATGTTTCAGCTTAGGTC

*PIN8R*: ATGGCACTACTCCTTGAGGCA

*LAT52F*: ATCGGGCCCACCGCGGTGGCGGCCGCAAGCTT

*LAT52R*: ATCGGATCCGCCTTAATCCTTTTTTTTCTTGTGTTTTTACTTCGGTC