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Supporting Online Material for

PIN Proteins Perform a Rate-Limiting Function in Cellular Auxin Efflux

Jan Petrášek, Jozef Mravec, Rodolphe Bouchard, Joshua J. Blakeslee, Melinda Abas, Daniela Seifertová, Justyna Wiśniewska, Zerihun Tadele, Martin Kubeš, Milada Čovanová, Pankaj Dhonukshe, Petr Skůpa, Eva Benková, Lucie Perry, Pavel Křeček, Ok Ran Lee, Gerald R. Fink, Markus Geisler, Angus S. Murphy, Christian Luschnig, Eva Zažímalová,* Jiří Friml

*To whom correspondence should be addressed. E-mail: eva.zazim@ueb.cas.cz

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Materials and Methods

Plant material, gene constructs, transformation and inducible expression

Arabidopsis seedlings were grown at a 16 hours light/8 hours dark cycle at 18-25 °C on 0.5 x MS with sucrose as described (*1*). The *XVE-PIN1* (Col-0) transgenic plants were obtained by introducing the $p_{G10\text{-}90}$*::XVE* activator and the *LexA::PIN1*; *LexA::GFP* reporter constructs (*2, 3*) into *pin1-7* mutant line. This line was crossed with *pgp1pgp19* double mutant (*4*) to generate *XVE-PIN1/pgp1pgp19* line. The *XVE-PIN1* construct was generated using *PIN1* cDNA (GenBank accession number AF089084). *GVG-PIN4,6,7* constructs were generated by inserting the corresponding cDNAs (*PIN4*: AF087016, *PIN6*: AF087819, *PIN7*: AF087820) into the pTA7002 vector (*5*). *GVG-PGP19:HA* construct was generated by introducing the full length genomic fragment of *PGP19* (locus name At3g28860) with C-terminal hemaglutinin tag (HA) into pTA7002 vector (*5*).

Cell suspension from *XVE-PIN1 Arabidopsis* line was established from calli induced on young leaves (*6*) and grown in liquid MS medium containing 1 μM 2,4-D. BY-2 tobacco cells (*Nicotiana tabacum* L., cv. Bright Yellow 2, (*7*)) were grown as described (*8*) and stably transformed by co-cultivation with *Agrobacterium* (*8*). Transgenic tobacco cells and calli were maintained on the media supplemented with 40 μg ml^{-1} hygromycin and 100 μg ml^{-1} cefotaxim. Expression of *PIN* and *PGP* genes in tobacco cells was induced by the addition of dexamethasone (DEX, 1 μM, 24 hours, except for stated otherwise) at the

beginning of the subcultivation period. The same approach was used for *Arabidopsis* cell culture, where 1 μM β-Estradiol (EST) was added. Both DEX and EST were added from stock solutions in DMSO (200 μM), appropriate volume of DMSO was added in controls.

Expression and localization analysis

Tobacco and *Arabidopsis* RNA was isolated using the Plant RNA Qiagen Mini-Prep and RT-PCR performed using Qiagen® OneStep RT-PCR or Invitrogen SuperSriptII kits according to the manufacturer's protocols.

Total protein fraction from *GVG-PGP19* tobacco cells was obtained after homogenization in liquid nitrogen using mortar and pestle. The frozen powder was then mixed with an equal volume of extraction buffer (50 mM Tris-HCl, pH 6.8; 2 % SDS; 36 % w/v urea; 30 % v/v glycerol; 5 % v/v mercaptoethanol; 0,5 % w/v Bromphenol Blue), vortexed for 1 min, boiled for 3 min, and centrifuged at 13,000 rpm and 4°C for 5 min. The supernatant was transferred into a new tube and re-centrifuged at 13,000 rpm and 4°C for 5 min. The resulting supernatant was defined as total protein extract and stored at –20°C until use.

Microsomal protein fraction from *GVG-PIN7* tobacco cells was used for immunoblot analysis of PIN7 protein. Briefly, cells were homogenized by sonication in extraction buffer (50mM Tris pH 6.8; 5% (v/v) glycerol; 1.5% (w/v) insoluble polyvinylpolypyrrolidone; 150mM KCl; 5mM Na EDTA; 5mM Na EGTA; 50mM NaF; 20mM beta-glycerol phosphate; 0.5% (v/v) solubilized casein, 1mM benzamidine; 1mM PMSF; 1μg/ml pepstatin; 1μg/ml leupeptin; 1μg/ml aprotinin; 1 Roche Complete Mini Protease Inhibitor tablet per 10ml). After centrifugation at 3,800 x *g* for 20 minutes, the supernatant was filtered through nylon mesh and spun again at 3,800 x *g*. The supernatant was centrifuged

at 100,000 x *g* for 90 min. The resulting pellet was homogenized and re-suspended in buffer containing 50mM Tris pH 7.5; 20% glycerol; 2mM EGTA; 2mM EDTA; 50-500µM DTE; 10µg/ml solubilized casein and protease inhibitors as in the extraction buffer. Equal amounts of protein (about 10µg) were heated at 60°C for 40 min in sample buffer (3% (w/v) SDS; 40mM DTE; 180mM Tris pH 6.8; 8M urea), and transferred on PVDF membrane using dot-blot (SCIE-PLAS, U.K.) or semi-dry electro-blot. Primary rabbit polyclonal anti-PIN7 antibody (*10*) or mouse monoclonal anti-HA antibody (Sigma) followed by secondary HRP-conjugated anti-rabbit antibody and ECL detection kit (Amersham Biosciences, U.K.) were used for dot or western blot analysis.

Indirect immunofluorescence method was used for immunolocalizations in *Arabidopsis* cell suspension (11) and BY-2 cells (*8*). Briefly, *Arabidopsis* cells were fixed for 30 min at room temperature with 4% (w/v) paraformaldehyde in 0.1 M PIPES, pH 6.8, 5 mM EGTA, 2 mM $MgCl_2$, and 0.4% (w/v) Triton X-100. Cells were then treated with the solution of 0.8% (w/v) macerozyme R-10 and 0.2% (w/v) pectolyase Y-23 in 0.4 M mannitol, 5 mM EGTA, 15 mM MES, pH 5.0, 1 mM PMSF, 10 µg/ml of leupeptin, and 10 µg/ml of pepstatin A. Then the cells were washed in PBS buffer and attached to poly-L-lysine coated coverslips and incubated for 30 min in 1% (w/v) BSA in PBS and incubated for 1 h with primary antibody. The specimens were then washed three times for 10 min in PBS and incubated for 1 h with secondary antibody. Coverslips with cells were carefully washed in PBS, rinsed with water with Hoechst 33258 (0,1µg/ml) and embedded in Mowiol (Polysciences) solution.

Tobacco BY-2 cells were pre-fixed 30 min in 100 µM MBS and 30 min in 3.7% (w/v) PFA in buffer consisting of 50 mM PIPES, 2 mM EGTA, 2 mM $MgSO_4$, pH 6.9, at 25°C and

subsequently in 3.7% (w/v) PFA and 1% Triton X-100 (w/v) in stabilizing buffer for 20 minutes. After treatment with an enzyme solution (1% (w/v) macerozyme and 0.2% (w/v) pectinase) for 7 min at 25°C and 20 minutes in ice cold methanol (at -20°C), the cells were attached to poly-L-lysine coated coverslips and treated with 1% (w/v) Triton X-100 in microtubule stabilizing buffer for 20 minutes. Then the cells were treated with 0.5% (w/v) bovine serum albumin in PBS and incubated with primary antibody for 45 minutes at 25°C. After washing with PBS, a secondary antibody in PBS was applied for 1 h at 25°C. Coverslips with cells were carefully washed in PBS, rinsed with water with Hoechst 33258 (0,1μg/ml) and embedded in Mowiol (Polysciences) solution.

The following antibodies and dilutions were used: anti- PIN1 (*13*, 1: 500), anti-PIN7 (*10*, 1:500), anti-HA (Sigma-Aldrich; 1:500), TRITC- (Sigma-Aldrich; 1:200), FITC- (Sigma-Aldrich; 1:200) anti-rabbit secondary antibodies. PIN immunostaining in yeast and HeLa cells was performed as described (*13, 14*).

All preparations were observed using an epifluorescence microscope (Nikon Eclipse E600) equipped with appropriate filter sets, DIC optics, monochrome integrating CCD camera (COHU 4910, USA) or colour digital camera (DVC 1310C, USA).

Quantitative analysis of root gravitropism

5 days old seedlings of WT-Col, *pgp1pgp19*, *XVE-PIN1* and *XVE-PIN 1/pgp1pgp19* lines grown vertically were transferred on new MS plates containing 4 μM β-estradiol for 12 hours. Seedlings were then stretched and plates turned through 135° for additional 12 hour gravity stimulation in dark. The angle of root tips from the vertical plane was measured using ImageJ software (NIH, USA). All gravistimulated roots were assigned to one of the

eight 45° sectors on gravitropism diagram. The length of bars represents the percentage of seedlings showing respective direction of root growth.

Auxin accumulation assays in plant, HeLa and yeast cells

Auxin accumulation experiments in suspension-cultured tobacco BY-2 cells were performed and the integrity of labeled auxins during the assay was checked exactly as described (*15, 8, 12*). The same protocol was used for suspension-cultured *Arabidopsis* cells. Labeled [^{3}H]IAA, [^{3}H]2,4-D and [^{3}H]Trp (specific activities 20 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO), and [^{3}H]NAA (specific radioactivity 25 Ci/mmol, Institute of Experimental Botany, Prague, Czech Republic) were used. Briefly, the accumulation was measured in 0.5-mL aliquots of cell suspension. Each cell suspension was filtered, resuspended in uptake buffer (20 mM MES, 40 mM Suc, and 0.5 mM $CaSO_4$, pH adjusted to 5.7 with KOH), and equilibrated for 45 min with continuous orbital shaking. Equilibrated cells were collected by filtration, resuspended in fresh uptake buffer, and incubated on the orbital shaker for 1.5 h in darkness at 25°C. [^{3}H]NAA was added to the cell suspension to give a final concentration of 2 nM. After a timed uptake period, 0.5-mL aliquots of suspension were withdrawn and accumulation of label was terminated by rapid filtration under reduced pressure on 22-mm-diameter cellulose filters. The cell cakes and filters were transferred to scintillation vials, extracted in ethanol for 30 min, and radioactivity was determined by liquid scintillation counting (Packard Tri-Carb 2900TR scintillation counter, Packard Instrument Co., Meriden, CT). Counts were corrected for surface radioactivity by subtracting counts obtained for aliquots of cells collected immediately after the addition of [^{3}H]NAA. Counting efficiency was determined by

automatic external standardization, and counts were corrected automatically. NPA was added as required from ethanolic stock solutions to give the appropriate final concentration. The concentration dependence of auxin accumulation in response to NPA or BFA was determined after a 20-min uptake period. For wash out experiments cells were loaded with [^{3}H]NAA (2 nM) for 30 min. After quick wash out, cells were re-suspended in fresh loading buffer but without [^{3}H]NAA; cell density before and after wash out was maintained the same. Relative NAA retention was measured as a radioactivity retained inside cells at particular time points after wash out and expressed as % of total radioactivity retained inside the cells just before wash out. The accumulation of various auxins or structurally related inactive compound (Trp) after induction of PIN or PGP expression was expressed together with SEs as the percentage of the accumulation of non-induced cells at time 30 min after application of respective labelled compound. If not indicated otherwise, 24 hours treatments with dexamethasone (1 μM) or β-estradiol (1 μM) were performed. Different sensitivities of PIN7- and PGP19-dependent [^{3}H]NAA efflux to NPA treatment (10 μM, 20 min) in *GVG-PIN7* and *GVG-PGP19:HA* cells was determined as the average value from three independent experiments. In each, the accumulation of [^{3}H]NAA was measured in NPA-treated induced and non-induced cells and scored after 20 minutes of incubation. The increase in the accumulation of [^{3}H]NAA upon NPA treatment in non-induced cells was considered as 100% and all other values expressed as the percentage of this increase.

The transient vaccinia expression system was used to transfect HeLa cells with PIN1:HA, PIN2:HA, and PIN7:HA in 6-well plates. The expression was verified by RT-PCR and western blot analysis. Auxin transport assays were performed exactly as described (*16, 14*). 16-24h after transfection cells were washed and incubated 40 min at 37°C, 5% CO_2 with

[^{3}H]IAA (26 Ci/mmol, Amersham Biosciences, Piscataway, NJ), or [^{3}H]BeA (20 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO). After incubation, cells were harvested, and retained radiolabeled substrate was quantitated. Net efflux is expressed as dpm/500,000 cells divided by the amount of auxin retained by cells transformed with empty vector minus the amount of auxin retained by cells transformed with gene of interest. Thus, the PIN-dependent decrease in retention is presented as positive efflux value expressed as means (n=3) with standard deviations. Cell viability after treatment was confirmed visually and via cell counting.

For auxin accumulation and growth assays in yeast, PIN2, PIN7 or PIN2:HA were expressed in *S. cerevisiae* strains *gef1* (*13*) and JK93da or *yap1-1* (*17*). The expression was verified by western blot analysis or immunolocalization. Export of [^{3}H]IAA (specific activity 20 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO) and [^{14}C]BeA (53 mCi/mmol, Moravek Biochemicals, Brea, CA) and growth assays were performed exactly as described (*17, 14*). The effluent species was determined by thin-layer chromatography of aliquots of exported [^{3}H]IAA (Supplementary fig. S4a) and images were taken using a phosphoimager (Cyclone, Packard Instruments) and by UV detection using [^{3}H]IAA as standard. Yeast viability before and after transport experiments was ascertained by light microscopy.

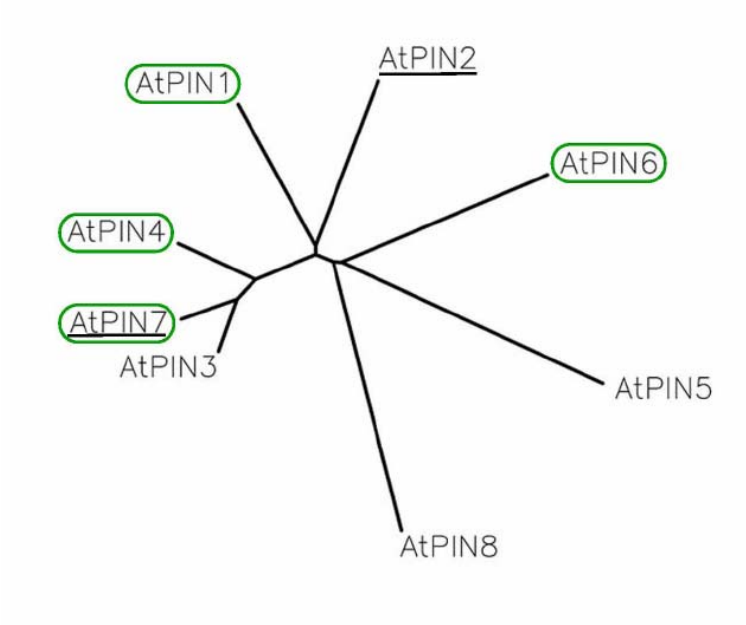

fig. S1 Arabidopsis *PIN* genes family.

Phylogenetic tree of 8 Arabidopsis *PIN* genes. Phenotypes of loss-of-function mutants in *PIN1*, *PIN2*, *PIN3*, *PIN4* and *PIN7* clearly suggest role in polar auxin transport and they all can be phenocopied by inhibitors of auxin transport (*18*). PIN6 remains functionally uncharacterized. PIN5 and PIN8 lack the middle hydrophilic domain and seem to by functionally distinct (*19*). Based on homology, *PIN7* is the most typical member of PIN family forming a distinct homologous subclade with *PIN3* and *PIN4*. *PIN6*, on the other hand, is the least homologous *PIN* from the *PIN1,2,3,4,6,7* subfamily. PIN1, PIN4, PIN6 and PIN7 (respective genes encircled in green) have been shown here to mediate auxin efflux *in planta*. PIN2 and PIN7 (genes underlined) show auxin efflux activity in heterologous systems. Notably, the confirmed expression of PIN1 in HeLa or yeast cells did not result in increased auxin efflux suggesting, in contrast to PIN2 and PIN7, that either PIN1 loses its functionality, when expressed in heterologous system, or distinctively PIN1 requires plant-specific factor(s) to mediate its function in auxin efflux.

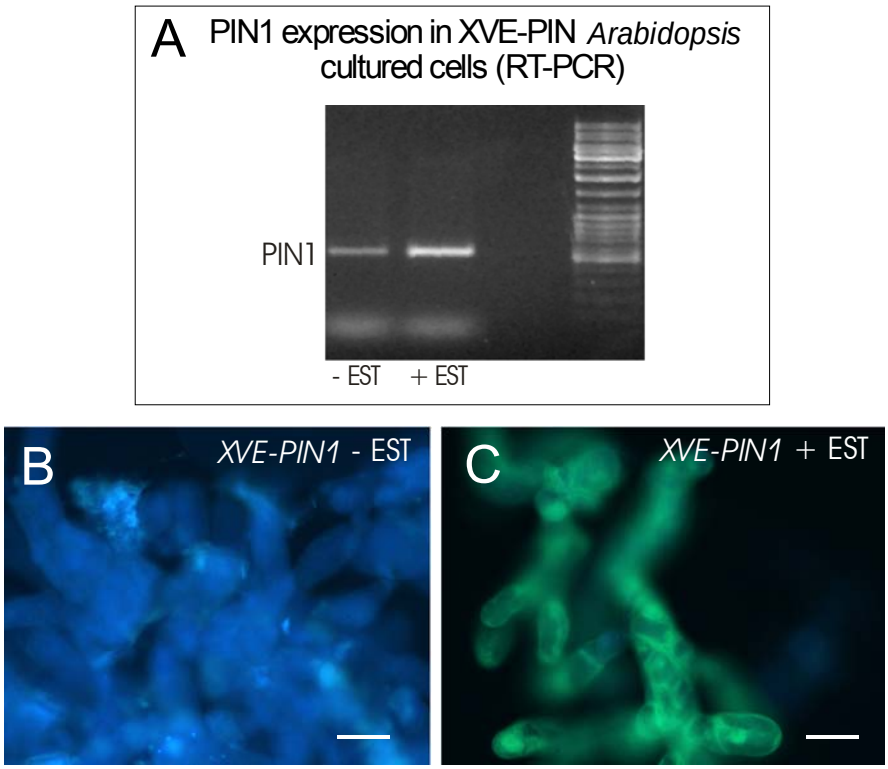

fig. S2 The expression of PIN1 in *XVE-PIN1 Arabidopsis* cultured cells

(A) RT-PCR of PIN1 in non-induced and β-estradiol-induced (1 μM, 24h) cells. (B, C) The activation of expression verified by the fluorescence of co-expressed GFP reporter. Compare the autofluorescence of cell walls in non-induced cells (B) with GFP fluorescence after 24 h incubation in 1 μM β-estradiol (C). Scale bars 30μm.

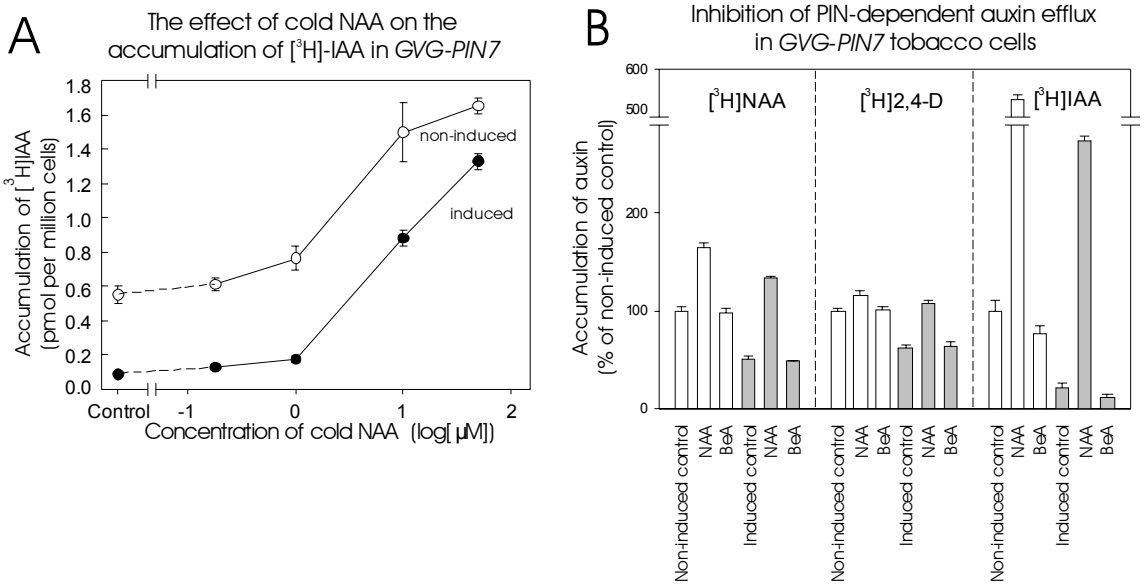

fig. S3 Auxin accumulation in *GVG-PIN7* BY-2 cells

(A) Displacement curve: The competitive inhibitory effect of cold (non-labeled) NAA on the accumulation of [^{3}H]IAA in non-induced and induced *GVG-PIN7* cells. (B) Effects of NAA and benzoic acid (BeA) on efflux of different auxins in DEX-treated (induced, full bars) and non-induced (open bars) *GVG-PIN7* cells. NAA (10 μM), a good substrate for auxin efflux machinery, interferes with both endogenous and PIN7-dependent efflux of [^{3}H]NAA, [^{3}H]2,4-D and [^{3}H]IAA in non-induced and induced *GVG-PIN7* cells, respectively. In contrast, structurally similar but inactive BeA (10 μM) does not have any detectable effect in the same experimental system.

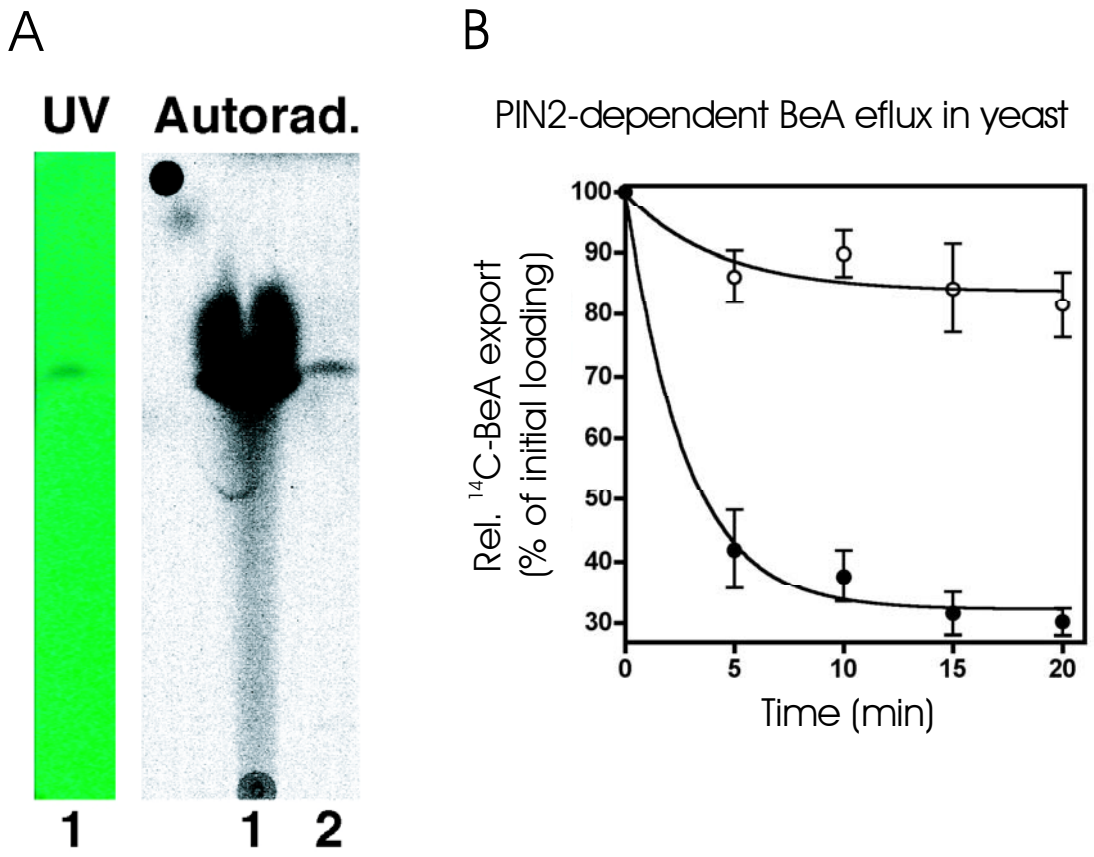

fig. S4 Control experiments for auxin efflux assays in yeast.

(A) The effluent species in yeast were determined to be [^{3}H]IAA by thin layer chromatography (lane 2). Non-exported [^{3}H]IAA was used as the standard which itself was verified by UV detection (lane 1). Images were taken using a phosphoimager (Cyclone, Packard Instruments) and by UV detection using [^{3}H]IAA as the standard. The integrity of exported [^{3}H]IAA in this assay was also proved by MS-MS, as described elsewhere (*14*).

(B) PIN2-expressing yeast show increased net efflux of [^{14}C]benzoic acid ([^{14}C]BeA)

compared to empty vector controls. [^{14}C]BeA (53 mCi/mmol, Moravek Biochemicals, Brea, CA) was used and transport experiments were performed exactly as described (*14*).

Supporting references and notes

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