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Recycling, clustering, and endocytosis jointly maintain PIN auxin carrier polarity at the plasma membrane

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Cell polarity reflected by asymmetric distribution of proteins at the plasma membrane is a fundamental feature of unicellular and multicellular organisms. It remains conceptually unclear how cell polarity is kept in cell wall-encapsulated plant cells. We have used super-resolution and semi-quantitative live-cell imaging in combination with pharmacological, genetic, and computational approaches to reveal insights into the mechanism of cell polarity maintenance in *Arabidopsis thaliana*. We show that polar-competent PIN transporters for the phytohormone auxin are delivered to the center of polar domains by super-polar recycling. Within the plasma membrane, PINs are recruited into non-mobile membrane clusters and their lateral diffusion is dramatically reduced, which ensures longer polar retention. At the circumventing edges of the polar domain, spatially defined internalization of escaped cargos occurs by clathrin-dependent endocytosis. Computer simulations confirm that the combination of these processes provides a robust mechanism for polarity maintenance in plant cells. Moreover, our study suggests that the regulation of lateral diffusion and spatially defined endocytosis, but not super-polar exocytosis have primary importance for PIN polarity maintenance.
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Introduction

Cell polarity is fundamental to many aspects of cell and developmental biology in both unicellular and multicellular organisms. In animals, the deposition of remarkably conserved polarity (Crumbs, Scribble and PAR) modules ensures the formation and maintenance of cell polarity (Tepass *et al*, 2001; Lu and Bilder, 2005; Humbert *et al*, 2006; Wells *et al*, 2006; Chen *et al*, 2010). Notably, the molecular components of animal polarity protein complexes are absent in plants (Geldner, 2009); thus, compared with animal cells, the underlying mechanisms that maintain cell polarity in plants are until now unknown.

In plants, PIN proteins are prominent polar cargos that determine the direction and rate of cellular export and intercellular transport of the plant growth substance auxin (Petrášek *et al*, 2006; Wiśniewska *et al*, 2006). The phytohormone auxin coordinates many growth and developmental processes in plants, which to a large extent is modulated via a dynamic control of cellular PIN polarity and its effects on directionality of auxin fluxes (Vanneste and Friml, 2009). At the molecular level, polar PIN targeting depends on cell type- and PIN sequence-specific factors (Wiśniewska *et al*, 2006). PIN proteins constitutively cycle between the plasma membrane and an endosomal pool (Geldner *et al*, 2001; Dhonukshe *et al*, 2007). The function of this cycling is unclear but it might serve as a mean to mediate polarity establishment and rapid polarity alteration (Friml *et al*, 2002; Dhonukshe *et al*, 2007; Kleine-Vehn *et al*, 2008). PIN internalization (endocytosis) from the plasma membrane depends on the clathrin machinery (Dhonukshe *et al*, 2007). In a positive feedback mechanism, auxin itself inhibits the clathrin-dependent endocytosis of PINs (Paciorek *et al*, 2005; Robert *et al*, 2010) and appears to contribute by this mechanism

to a self-organizing, auxin-mediated tissue polarization (Sachs, 1981; Scarpella *et al*, 2006; Wabnik *et al*, 2010).

Mechanisms underlying apical and basal polar PIN deposition appear to be molecularly distinct (Kleine-Vehn *et al*, 2006; Wiśniewska *et al*, 2006) and recruitment into these pathways depends on the phosphorylation status of PIN proteins (Friml *et al*, 2004; Kleine-Vehn *et al*, 2009; Dhonukshe *et al*, 2010; Huang *et al*, 2010). The serine/threonine protein kinase PINOID (PID) catalyzes PIN phosphorylation (Michniewicz *et al*, 2007), leading to basal-to-apical polarity switches by PIN recruitment to an ARF-GEF GNOM-independent pathway (Kleine-Vehn *et al*, 2009). The phosphatase PP2A counteracts PINOID action on PIN phosphorylation and preferentially promotes GNOM-dependent basal PIN localization (Michniewicz *et al*, 2007; Kleine-Vehn *et al*, 2009).

Besides PIN-dependent auxin transport mechanism, polar vesicle trafficking also contributes to other often diverse cellular functions, such as polar tip growth, nutrient uptake, root soil interface establishment, and pathogen response (Kwon *et al*, 2008; Takeda *et al*, 2008; Alassimone *et al*, 2010; Takano *et al*, 2010; Łangowski *et al*, 2010).

Despite this wealth of molecular clues into polar targeting and subcellular dynamics of PIN proteins, the knowledge on cellular mechanisms underlying the establishment and maintenance of polar distribution of PIN proteins or other polar cargos in plant cells are still limited. It seems that initial secretion of newly synthesized PIN proteins is non-polar and their polar distribution is established only in the next step by endocytic recycling (Dhonukshe *et al*, 2008), but it is entirely unclear, how, once established, the polar PIN distribution is maintained within fluid plasma membrane environment. Establishing semi-quantitative and subdiffraction resolution fluorescence imaging for living plant cells have provided us with unexpected insights into the mechanisms underlying dynamic maintenance of PIN polarity. We illustrate (i) presumably TGN/endosome guided super-polar targeting of PIN proteins to the center of polar domains, (ii) PIN recruitment to immobile membrane clusters that reduce lateral PIN mobility and (iii) PIN protein retrieval at the lateral cell side by spatially defined clathrin-dependent endocytosis. *In silico* model simulations are consistent with these experimental observations and reveal the individual roles of these cellular processes in the organization of sharply defined polar plasma membrane domains.

Results

Evaluation of PIN polarity establishment in plant cells

In order to obtain further insight into the polar targeting in plants, we investigated prominent polar plant cargos of the PIN phytohormone auxin efflux carrier family (Figure 1A). In root epidermal cells, PIN2 proteins localize predominantly to the apical plasma membrane domain, but to a lesser extent also to other sides of the cell (Supplementary Figure 1A–C). We applied a semi-quantitative confocal microscopy technique to visualize relative fluorescence intensity of PIN2–GFP (Figure 1A) or endogenous PIN2 (Figure 1B), enabling us to address the ratio of polar PIN distribution within the plasma membrane. 3D imaging (x,y,z) combined with color-coded fluorescence intensity profiling revealed that the majority of PIN2 reporter indeed localized to the apical cell side with a remarkably steep decrease in intensity at the edges of the apical domain (Figure 1A and B).

Preferential PIN1 and PIN2 targeting to the center of the polar plasma membrane domain

To address the so far elusive mechanism of cell polarity maintenance in plant cells, we initially analyzed polar recruitment of PIN2 in the apical cell side. We performed z-stack imaging (0.5 μm steps) of whole root epidermal cells and calculated 3D projections to obtain a detailed representation of PIN2–GFP fluorescence intensity within the apical cell side. Highest PIN2–GFP fluorescence intensities could be detected in an inner core of the apical plasma membrane that we have designated the super apical domain (Figure 1C). Endogenous PIN2 proteins also displayed localization predominantly restricted to the super apical domain (Figure 1E). Notably, this super-polar PIN2 localization was not observed in all epidermal cells and appears to be less pronounced in younger epidermal root cells (Figure 1E).

Next, we investigated PIN2:PIN1–GFP2 transgenic lines that preferentially show basal PIN1 localization in root epidermal cells (Wiśniewska *et al*, 2006). The strongest PIN1–GFP2 localization was present in the inner core of the basal cell side (Figure 1D). In contrast, non-polar plasma membrane marker BRI1–GFP was not enriched in the basal or apical cell sides (Supplementary Figure 1H), indicating specific polar targeting mechanisms for PIN proteins.

To address whether super-polar PIN2 targeting is linked to its enhanced delivery to the apical cell side, we photobleached the entire apical cell side and recorded its recycling-based recovery within 15–30 min. In the majority of cells (n=33; 66%) displaying super-polar PIN2 localization stronger PIN2 recovery in the inner core of the apical cell side was observed (Figure 1G; Supplementary Figure 2). This finding suggests that super-polar PIN2 localization requires a defined polar exocytosis/delivery mechanism.

Notably, PIN cargos containing endomembranes (hereafter defined as endosomes) are frequently observed beneath the plasma membrane. PIN2–GFP signal enrichment in the apical plasma membrane correlates with a frequent endosomal occurrence beneath (Figure 1F). One could speculate that endosomal positioning beneath the plasma membrane and subsequent spatially defined exocytosis or fusion could enable highly defined 'super-polar' delivery of PIN proteins (Figure 1F; Supplementary Figure 1D–F).

Super-polar PIN delivery is not sufficient for defined PIN polarity maintenance

To test whether super-polar delivery of PIN proteins to the polar domain is sufficient to explain realistic PIN polarity pattern, we used computer model simulations (Figure 1I–L, for detailed model description, see Supplementary information).

The apical and neighboring lateral sides of root epidermis cell were modeled explicitly. We represented the plasma

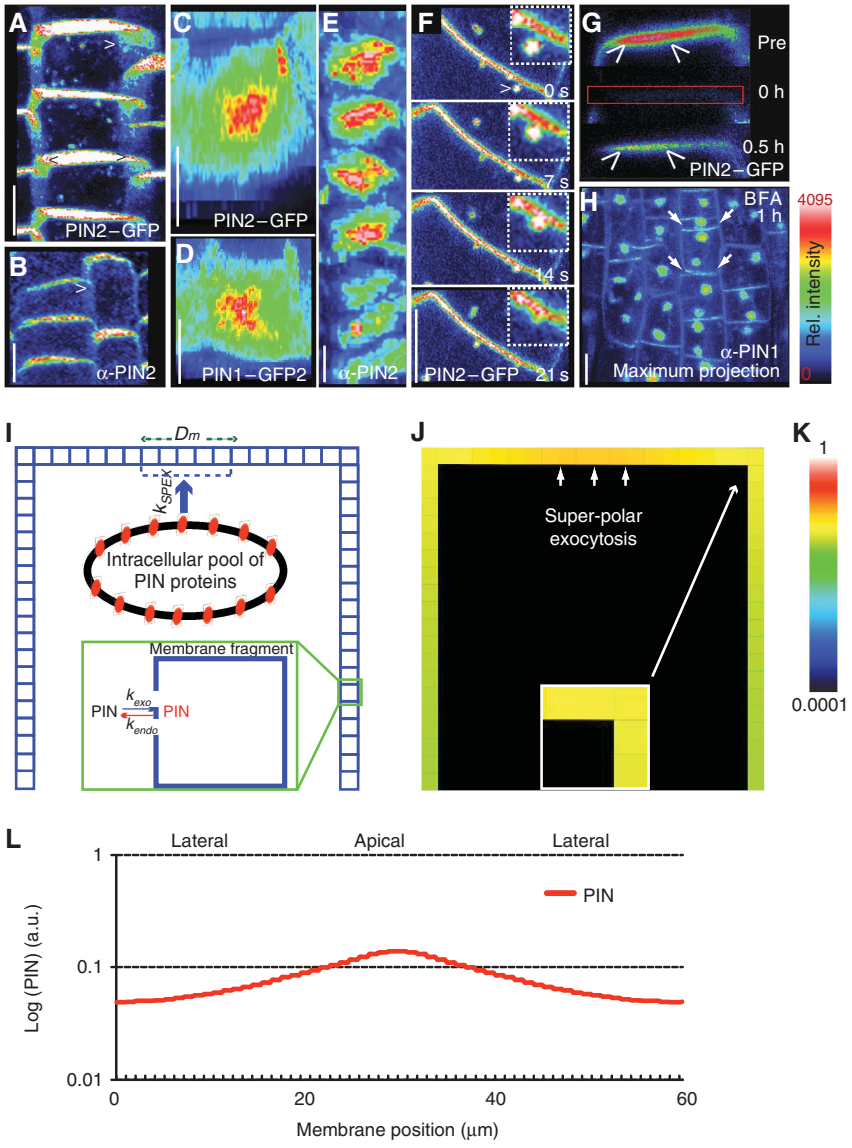


Figure 1 Super-polar PIN2 targeting. (**A**, **B**) Maximum projection of epidermal PIN2–GFP (12 sections at 1 μm step size) (A) and endogenous PIN2 (6 sections at 2 μm step size) (B) reveals preferential apical PIN2 localization and steep decrease at the lateral cell side (see arrowheads). (**C**–**E**) Top view of the apical cell surface by 3D xyz projection (30 sections at 0.5 μm step size) of an epidermal cell expressing PIN2:PIN2–GFP (C) and PIN2:PIN1–GFP2. Apical PIN2–GFP and basal PIN1–GFP2 signal intensity is highest in the center and gradually gets weaker toward the cell edges. (D, E) PIN2 antibody staining depicts seven consecutive cells (45° angle). (**F**) Time-lapse (7 s interval) of PIN2–GFP containing endosome at the apical cell surface (see arrowhead and inset). (**G**) Medial, single scan sections of PIN2–GFP expressing root epidermal cells. Pre-bleached cell shows preferential super-polar PIN2 localization and preferential super-polar recovery (0.5 h) after photobleaching (both depicted by arrowheads; see also Supplementary Figure 2). (**H**) BFA (50 μM) treatment for 1 h results in PIN1 (antibody) accumulation in BFA compartments in root stele cells (z-stacks and maximum projection). BFA compartments reside close to the basal or apical cell side. Arrows indicate preferential polar distribution of PIN1 at the basal cell side. (**I**) Schematics of the computer model. Intracellular space is abstracted by a single endosomal compartment that contains the common intracellular pool of PIN proteins. PIN proteins recycle between the endosomal compartment and each discrete plasma membrane fragment at the constant basal PIN turnover rates k_{exo} and k_{endo} (blue and red arrows). PIN proteins are delivered by the super-polar exocytosis (k_{SPEX}; thick blue arrow) to the center of the polar plasma membrane domain. D_m describes the free lateral diffusion of PIN proteins in the plasma membrane. (**J**–**L**) Computer model simulations with spatially restricted polar delivery of PIN proteins (I, white arrow) predicted the loss of apical PIN polarization in root epidermis cell due to lateral diffusion (Supplementary information; Supplementary Movie 1). (K) Color coding scheme for model simulations (log scale). (L) Steady-state PIN distribution pattern. Graph depicts nearly uniform distributions of PIN proteins in all cell sides. Fluorescence intensity from 0 (black) to 4095 (brightest/white) is represented by the color code shown next to the figure (A–H). Scale bar: 5 μm.

membrane as a sequence of discrete membrane fragments each of 1×1 micron size (Figure 1I). Each fragment was associated with either the apical or one of the two lateral cell sides (Figure 1I). The intracellular membranes were approximated by one single endosomal compartment that represented the common intracellular pool of PIN proteins (Figure 1I). The redistribution of PIN proteins between membrane fragments and endosomal compartments was determined by the PIN turnover rates (k_{exo} and k_{endo}) (Figure 1I, thin blue and red arrows). This basal exocytosis and endocytosis rates are set to

be constant for all cell sides. We considered that PIN proteins display lateral diffusion (D_m) within the plasma membrane (Figure 1I).

The super-polar delivery of PIN proteins occurs to a central region within one side of the cell, presumably via endosomal trafficking mechanisms and subsequent spatially defined protein recycling. We modeled this process by assuming an increased rate of PIN delivery (k_{SPEX}) to the center of the polar domain (Figure 1I, thick blue arrow). However, the assumption of a highly defined, super-polar PIN deposition did not lead to a pronounced PIN polarization in our model (Figure 1J–L). In accordance, the pharmacological inhibition of GNOM-dependent PIN1 exocytosis to the basal cell side did not lead to the total loss of preferential basal PIN1 localization (Figure 1H; Supplementary Figure 1G). These findings indicate that polar PIN delivery is not sufficient to explain the dynamic pattern of PIN polarization.

PIN proteins display reduced lateral diffusion within the plasma membrane

Once proteins are deposited at the plasma membrane, the fluidity of the membrane allows their lateral diffusion. Notably, polar-competent PIN proteins have been suggested to display reduced lateral diffusion in the plasma membrane compared with non-polar markers, such as PLASMA MEMBRANE INTRINSIC2 (PIP2) or LOW-TEMPERATURE-INDUCED6b (LTI6) (Dhonukshe *et al*, 2008; Men *et al*, 2008).

We utilized the combination of fluorescence recovery after photobleaching (FRAP; Chen *et al*, 2006) and a confocal-based semi-quantitative imaging approach to address lateral diffusion of plasma membrane proteins. PIN1 and PIN2 short-term, diffusion-based recovery in root epidermal cells was visibly weaker than LTI6b–GFP recovery (Figure 2A–C). The fluorescence PIN2–GFP recovery was largely abolished after the whole plasma membrane was bleached (n=15; mean recovery: 4.1%; s.d.: 1.8%), indicating that recovery within 2 min was due to lateral diffusion and is largely independent of secretion. This finding is in agreement with previous inhibitor-based demonstration of lateral diffusion of plasma membrane proteins (Men *et al*, 2008). Next, we analyzed the kinetics of PIN2 and LTI6b fluorescent recovery. The GFP–LTI6b showed rapid (maximum of 76%) recovery of initial fluorescence within 2 min after photobleaching (Figure 2D), whereas the recovery of PIN2–GFP was only about 14% (Figure 2C and D), suggesting that only a small fraction of the PIN proteins can freely diffuse laterally within the plasma membrane, whereas the majority appears to be non-mobile. Notably, the lateral diffusion of PIN2 was not only reduced in the center of the apical cell side (n=27; mean

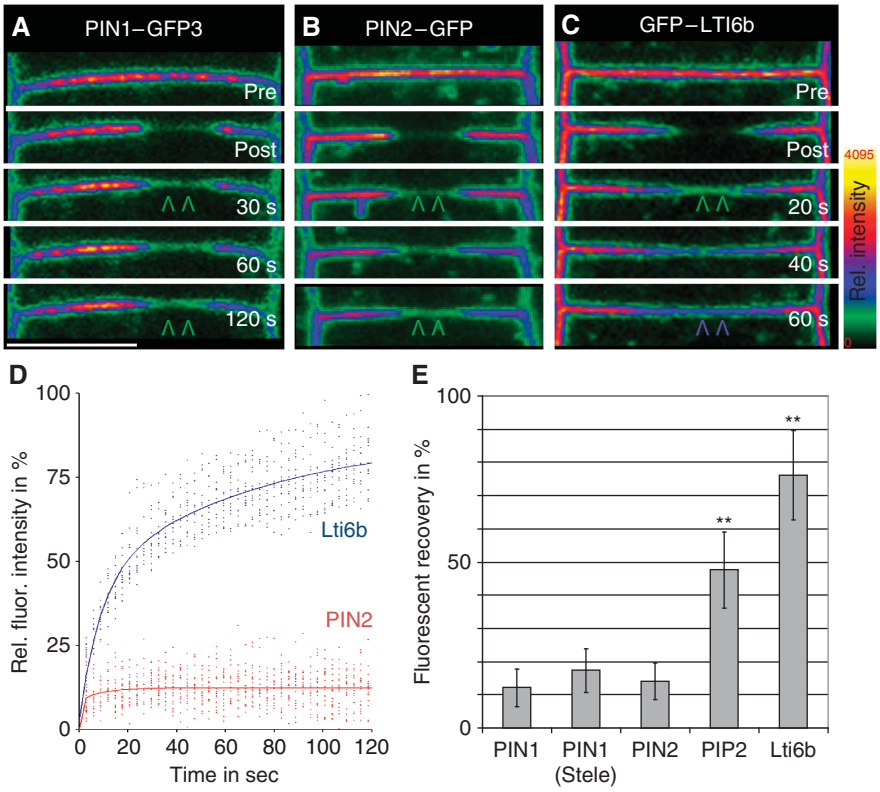


Figure 2 PIN proteins reside in a non-mobile membrane fraction. (**A**–**C**) pPIN2:PIN1–GFP3 (A) or pPIN2:PIN2–GFP (B) displays only weak fluorescence recovery after photobleaching (FRAP) within 2 min as visualized by color-based fluorescence intensity coding. In contrast, the non-polar plasma membrane marker GFP–LTI6b (C) displays rapid and substantial recovery within 1 min. (**D**, **E**) Fluorescence recovery kinetics of PIN2–GFP and GFP–LTI6b FRAP experiment (D). Maximum fluorescence recoveries of the non-polar plasma membrane markers PIP2–GFP (47.6%; n=23) and LTI6b–GFP (76.1%; n=20 cells) are significantly higher (E) than is the recovery of epidermal PIN2–GFP (14%; n=27 cells) and PIN2:PIN1–GFP3 (12.1%; n=12) and PIN1:PIN1–GFP (17.3%; n=14) in stele (**indicate $P<0.001$). Error bars represent standard deviation. Fluorescence intensity from 0 (black) to 4095 (brightest) is represented by the color code shown next to the figure (A–C). Scale bar: 5 μm. Source data is available for this figure in the Supplementary Information.

recovery: 14.0%; s.d.: 5.6%), but also in the peripheral sides of the apical cell side (n=11; mean recovery: 12.7%; s.d.: 5.3%; P-value: 0.76).

Similar to PIN2, PIN1 in root epidermal and stele cells showed severely reduced lateral diffusion (Figure 2E), indicating a general mechanism for PIN protein immobilization. The aquaporin PIP2;1 had a relatively high non-mobile fraction in the plasma membrane (Figure 2E). Although the lateral mobility of PIP2;1 was significantly higher than that of PIN2, but lower than Lti6b (Figure 2E), indicating complex regulation of lateral diffusion of different plasma membrane proteins.

Our data suggest that the reduced lateral diffusion behavior of PIN proteins (Dhonukshe *et al*, 2008; Men *et al*, 2008) might not be due to slower diffusion rates, but rather relate to a mechanism that immobilizes a large fraction of PIN proteins in the plasma membrane.

PIN proteins localize to membrane clusters within the plasma membrane

Reduced lateral PIN mobility within the plasma membrane might be regulated by membrane heterogeneity; therefore, we analyzed the PIN protein distribution within the plasma membrane.

Semi-quantitative confocal and super-resolution microscopy revealed that PIN1 and PIN2 auxin efflux carriers are not evenly distributed in the plasma membrane but that they accumulate in distinct 'clusters' (Figure 3A and B). Similarly,

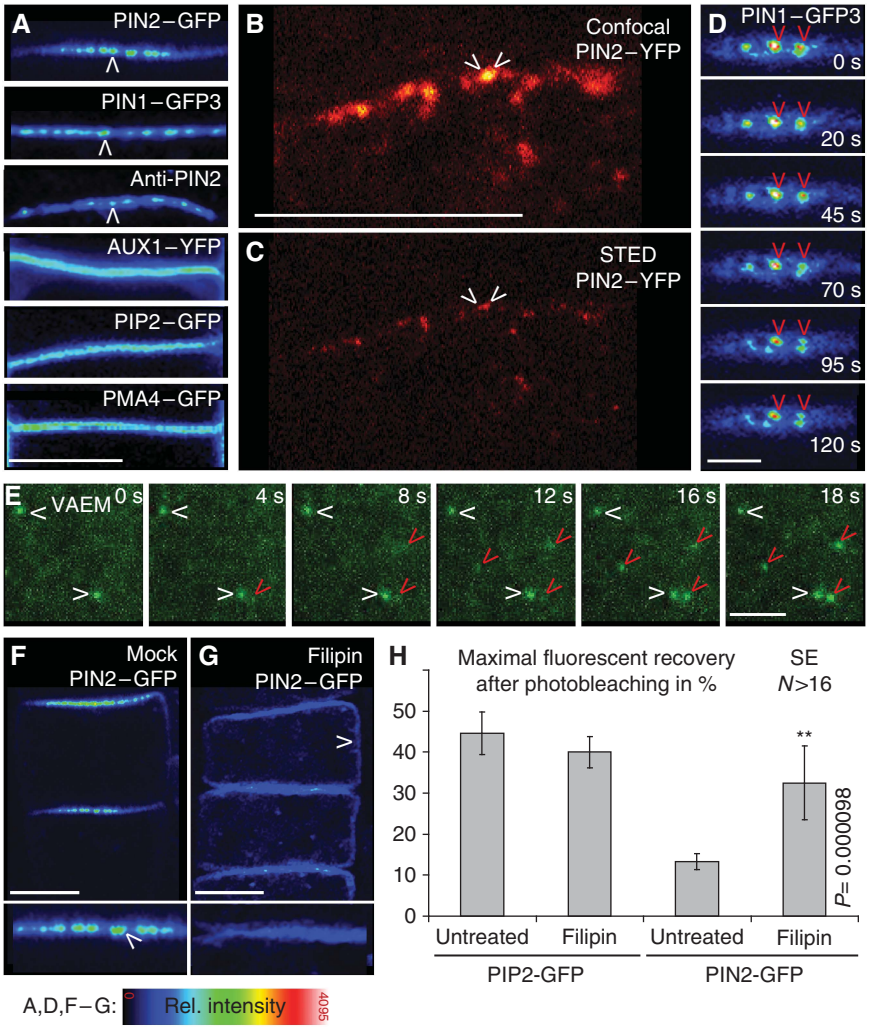


Figure 3 PIN proteins reside in non-mobile membrane clusters. (**A**) PIN proteins, but not non-polar AUX1, PIP2, or PMA4, are localized in membrane clusters (arrowheads). (**B**, **C**) Confocal (B) and super-resolution STED (C) microscopy of PIN2-YFP reveal PIN2 clusters in the apical plasma membrane (arrowheads) (see also Supplementary Figure 3 on cluster size evaluation). (**D**) pPIN2:PIN1–GFP (in epidermal cells) is non-mobile within membrane clusters (arrowheads) during 2 min time-lapse imaging (see also pPIN2:PIN2–GFP kymograph representation of 10 min time lapse in Supplementary Figure 4). (**E**) VAEM imaging revealed non-mobile PIN2–GFP clusters (white arrowheads) and the gradual appearance (red arrowheads) of PIN2–GFP at the outer lateral plasma membrane of root epidermal cells. (**F**, **G**) PIN2–GFP localization in membrane clusters (arrowhead) (F) is reduced after filipin (100 μM, 3 h) treatment (arrowhead depicts loss of PIN2 polarity) (G) (see also images with variable gain settings in Supplementary Figure 4). (**H**) Filipin treatment (100 μM; 20 min) results in a significant increase in membrane lateral mobility of PIN2–GFP, but not of PIP2;1–GFP ($n>16$ cells; **indicates $P<0.0001$). Error bars depict standard error. Fluorescence intensity from 0 (black) to 4095 (brightest) is represented by the color code shown beneath the figure (A, D, F–G). Scale bar: 5 μm. All images are single scans and within the dynamic range of fluorescent detection. Source data is available for this figure in the Supplementary Information.

the basal cargo PIN1 resided in plasma membrane clusters in root stele cells (Supplementary Figure 1I). In contrast, the auxin influx carrier AUX1 or other non-polar cargos, such as LTI6b, PIP2;1, Brassinosteroid receptor BRI1, or plasma membrane H^+-ATPase PMA4 showed a very weak heterogeneity, but we did not detect any comparable clustering as for PIN proteins (Figure 3A; Supplementary Figure 1H). To improve resolution and to elaborate on the specificity of this finding, we employed subdiffraction resolution STED microscopy (Hell and Wichmann, 1994; Willig *et al*, 2006). While this method has been applied to mammalian cells before, here we established this method for the imaging of living plant cells. We observed PIN2-YFP accumulation in membrane clusters (Figure 3B and C), but we did not detect any AUX1-YFP proteins in plasma membrane subdomains (Supplementary Figure 3A and B). Furthermore, STED microscopy revealed that the PIN2-containing membrane clusters vary on average between 100 and 200 nm in diameter (Supplementary Figure 3C and D).

These data illustrate that PIN proteins are likely distributed in distinct subdomains (clusters) in the nanometer range in the plasma membrane. These observations are in good accordance with our findings that PIN proteins reside in two distinct pools within the plasma membrane (majority in a relatively immobile and to a lesser extent in a mobile fraction).

Membrane clusters are non-mobile and might reduce lateral diffusion of PIN proteins

Next, we analyzed whether PIN clustering could be linked to the reduced lateral diffusion of PIN proteins in the plasma membrane. Remarkably, PIN1 and PIN2 proteins associated with clusters were largely non-mobile in the time window of at least 10 min (Figure 3D; Supplementary Figure 4A). Variable angle epifluorescence microscopy (VAEM) revealed that also PIN2 proteins at the lateral cell side displayed non-mobile clustering in the plasma membrane (Figure 3E). In addition to immobile existing clusters at the lateral cell side, new PIN2 clusters appeared (Figure 3E), indicating either PIN protein delivery or recruitment to the membrane clusters. The gradual appearance of PIN2 clusters at the lateral cell side rather suggests a gradual recruitment scenario of free PIN2 proteins into membrane clusters at the lateral cell side, possibly reducing the lateral PIN2 diffusion.

In yeast, plasma membrane compartmentalization partially depends on its sterol composition (Bagnat and Simons, 2002; Grossmann *et al*, 2008); however, the lateral diffusion of PIN2 was not affected in the sterol mutant *cpi* (Men *et al*, 2008). Sterol-dependent cell functions can be furthermore studied using the sterol-binding agents filipin and cyclodextrin which cause sterol desorption (Zidovetzki and Levitan, 2007) and modulates plant plasma membranes (Kleine-Vehn *et al*, 2006; Men *et al*, 2008). Filipin treatments reduced the heterogeneity of PIN2–GFP labeling in the plasma membrane (Figure 3F and G; Supplementary Figure 4B and C) and the polar localization of PIN2 after prolonged disruption of membrane sterols (Figure 3G).

Short-term filipin treatment (20 min) did not visibly affect the PIN protein amount in the apical plasma membrane, but was sufficient to enhance the fluorescence recovery rate of PIN proteins from 13 to 32% (Tukey test, $P<0{,}01$; Figure 3H), but not that of the non-polar plasma membrane marker PIP2;1 (Figure 3H).

These findings suggest that the clustering of PIN proteins in the plasma membrane largely contributes to limiting lateral PIN diffusion in the plasma membrane and possibly to the maintenance of PIN polarity.

Super-polar PIN deposition and reduced lateral diffusion are not sufficient for PIN polarity maintenance

The reduced lateral diffusion of PIN proteins (Dhonukshe *et al*, 2008; Men *et al*, 2008) might not rely on slower diffusion rates, but, in contrast, on immobilization of a large fraction of PIN proteins in the plasma membrane. To evaluate this assumption and to test its potential importance for PIN polarity maintenance, we integrated this reduced PIN diffusion mechanism into our computational model approach (Figure 4A–C).

Intriguingly, the simulated combination of super-polar exocytosis (Figure 1H) and reduced lateral diffusion due to PIN clustering in the membrane (Figure 4A) led to the dynamic maintenance of PIN polarization at the apical cell side (Figure 4B). These findings are in accordance with our experimental findings and indicate that lateral diffusion is an important parameter for PIN polarity maintenance (Figure 2). However, compared with the experimental data (Figure 1A and B), model simulations did not predict a steep decrease in PIN protein distributions at the lateral cell side (Figure 4C). Similarly, simulations of highly pronounced super-polar exocytosis of PIN proteins, reduced lateral PIN diffusion (Supplementary Figure 5A–H), or their combination (Supplementary Figure 5I and J) did not result in realistic, polar PIN distribution patterns (Figure 1A and B). Based on these *in silico* simulations, we suggest that super-polar PIN deposition and reduced lateral mobility are not sufficient to explain PIN polarity maintenance in plant cells.

Spatially defined clathrin-dependent PIN endocytosis is required for the maintenance of PIN polarity

The combination of super-polar deposition and reduced lateral mobility might not be sufficient for the observed steep decrease in intensity of PIN2–GFP at the lateral cell sides (Figures 4A, B and 1). Therefore, we assume that additional PIN retrieval mechanisms specifically at the lateral cell side might contribute to the regulation of PIN polarity maintenance. Hence, we subsequently investigated the requirement of endocytosis for the dynamic PIN polarity maintenance.

PIN protein internalization is largely dependent on the clathrin machinery (Dhonukshe *et al*, 2007). To assess the spatial occurrence of clathrin, we examined clathrin light chain (CLC)–GFP localization at the plasma membrane by semi-quantitative confocal imaging. Interestingly, CLC–GFP has a stronger localization to the lateral cell sides as compared to the apical and basal sides (Figure 5A). This clathrin enrichment at lateral cell sides appeared even more pronounced after the onset

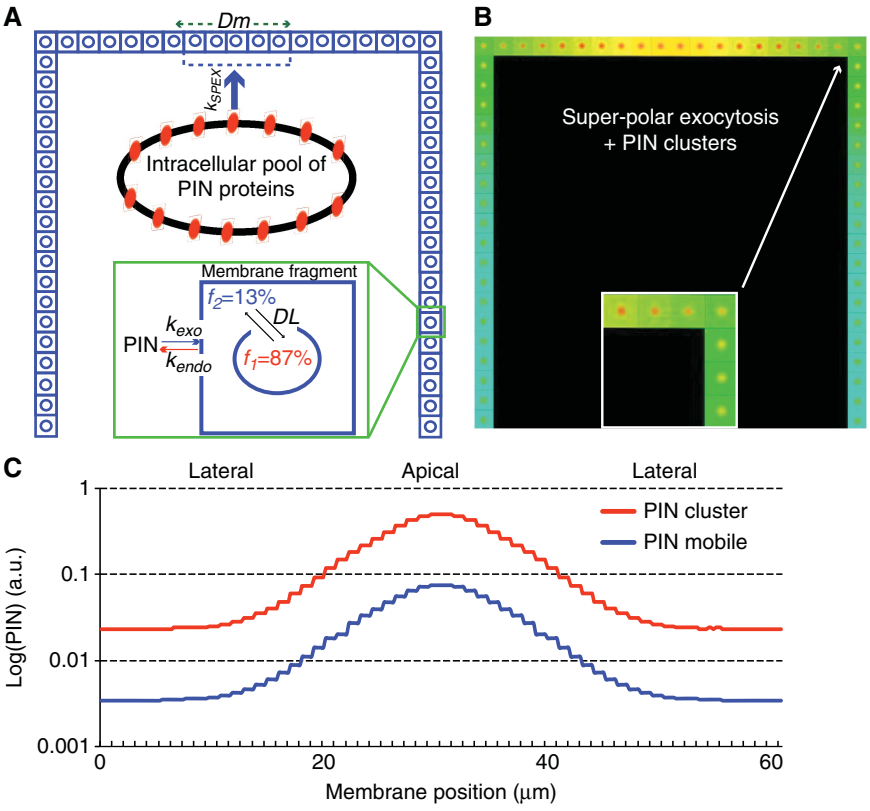


Figure 4 Integration of super-polar PIN delivery and membrane-associated PIN clusters for PIN polarity maintenance. (**A**) Schematics of the extended computer model. Inside each discrete membrane fragment PIN proteins are distributed in two groups (D_L) with non-mobile (f_1) and mobile (f_2) pool. Only the PIN proteins within the mobile pool (f_1) diffuse (D_m) to the adjacent membrane fragments. The remaining model assumption is as in Figure 1H. (**B**, **C**) A weak PIN polarization was predicted by computer model simulations assuming super-polar delivery of PIN proteins and PIN immobilization in membrane clusters (B) (see also Supplementary information and Supplementary Movie 2). However, pronounced leakage of PIN proteins toward cell junctions was observed (white arrow). (C) Steady-state PIN distribution in the plasma membrane showed a shallow gradient-like profile. Color coding is the same as in Figure 1K.

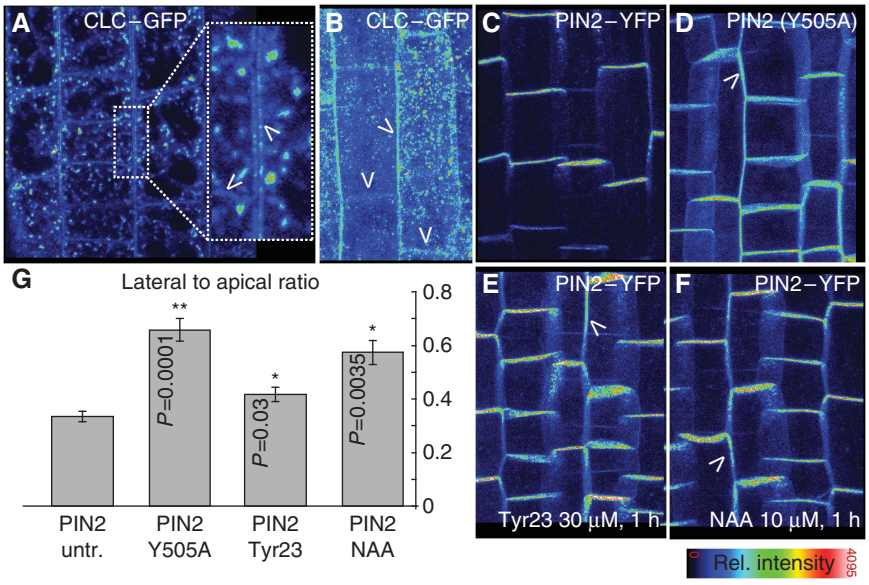


Figure 5 Clathrin-dependent PIN internalization at the lateral cell side for polarity maintenance. (**A**, **B**) Single scan (A) and 3D maximum projection (B) of clathrin light chain-GFP (CLC–GFP). Stronger clathrin activity occurs at the lateral cell side than at apical and basal sides, especially after onset of elongation (B). Arrowheads indicate basal and lateral cell margins. (**C**–**F**) Maximum projections depict polar localization of PIN2-YFP to the apical cell side (C). Polar localization is affected in tyrosine motif mutation *PIN2*^{Y505A}*-YFP* (D), or after short-term tyrphostinA23 (30 μM; 1 h) (E) and NAA (10 μM; 1 h) (F) treatments. Arrowheads indicate enhanced PIN2-YFP localization to the lateral side of the cell. (**G**) Gray value-based evaluation of lateral PIN2-YFP localization using lateral to apical ratio measurements (n=10; measured in the transition zone). Fluorescence intensity from 0 (black) to 4095 (brightest) is represented by the color code shown beneath the figure (A–F). Scale bar: 5 μm. Source data is available for this figure in the Supplementary Information.

of cellular elongation (Figure 5B). This finding indicates that plant cells can differentially modulate the activity of clathrin at different cell sides, which could contribute to PIN polarity maintenance.

To address whether spatio-temporal clathrin activity could directly influence PIN polarity, we selectively affected the clathrin-dependent PIN internalization by mutating a conserved tyrosine residue of PIN2 that is presumably required for its cargo-specific sorting into clathrin-coated pits (Supplementary Figure 6). While the majority of *PIN2*^{Y505A}*-YFP* still localized to the apical cell side in root epidermal cells, the mutation strongly enhanced PIN2 localization to the lateral cell side (Figure 5C, D, and G). Notably, *pPIN2:PIN2*^{Y505A}*-YFP* showed reduced PIN internalization and failed to fully rescue the *pin2* mutant phenotype (Supplementary Figure 6). These findings suggest that clathrin function is required for PIN polarity maintenance, possibly via PIN protein retrieval specifically from the lateral cell side.

Next, we invoke the temporal inhibition of the clathrin machinery by Tyrphostin23 treatments that affect tyrosine motif-dependent cargo recruitment to the clathrin-coated pit (Dhonukshe *et al*, 2007). The short-term treatment with Tyrphostin23 enhanced lateral PIN2 localization (Figure 5E and G). Notably, PIN2 mislocalization is apparent with an apical-to-basal gradient in fluorescence intensity at the lateral cell side (Figure 5E and G). This is presumably the result of slow lateral diffusion of PIN2-YFP from the apical cell side. Notably, short-term Tyrphostin23 treatment had the most pronounced effect after the onset of cellular elongation.

Auxin itself negatively affects clathrin-dependent PIN endocytosis in a transient manner (Paciorek *et al*, 2005; Robert *et al*, 2010) and also enhanced PIN2 residence at the lateral cell side after 1–2 h of exogenous application (Figure 5F and G). This provides a mechanistic possibility that auxin affects polar PIN2 localization in root epidermal cells by spatial inhibition of clathrin-dependent endocytosis (Robert *et al*, 2010; Wabnik *et al*, 2010). Auxin perception in the extracellular space has been suggested to feedback on PIN polarity (Wabnik *et al*, 2010). In such a mechanism directional PIN-dependent auxin efflux could enhance PIN polarization and could explain the appearance of PIN protein gradients in the lateral cell side. In summary, independent genetic and pharmacological approaches revealed that clathrin-dependent PIN endocytosis at the lateral cell side is required for dynamic PIN polarity maintenance.

Interweaving mechanism for PIN polarity maintenance

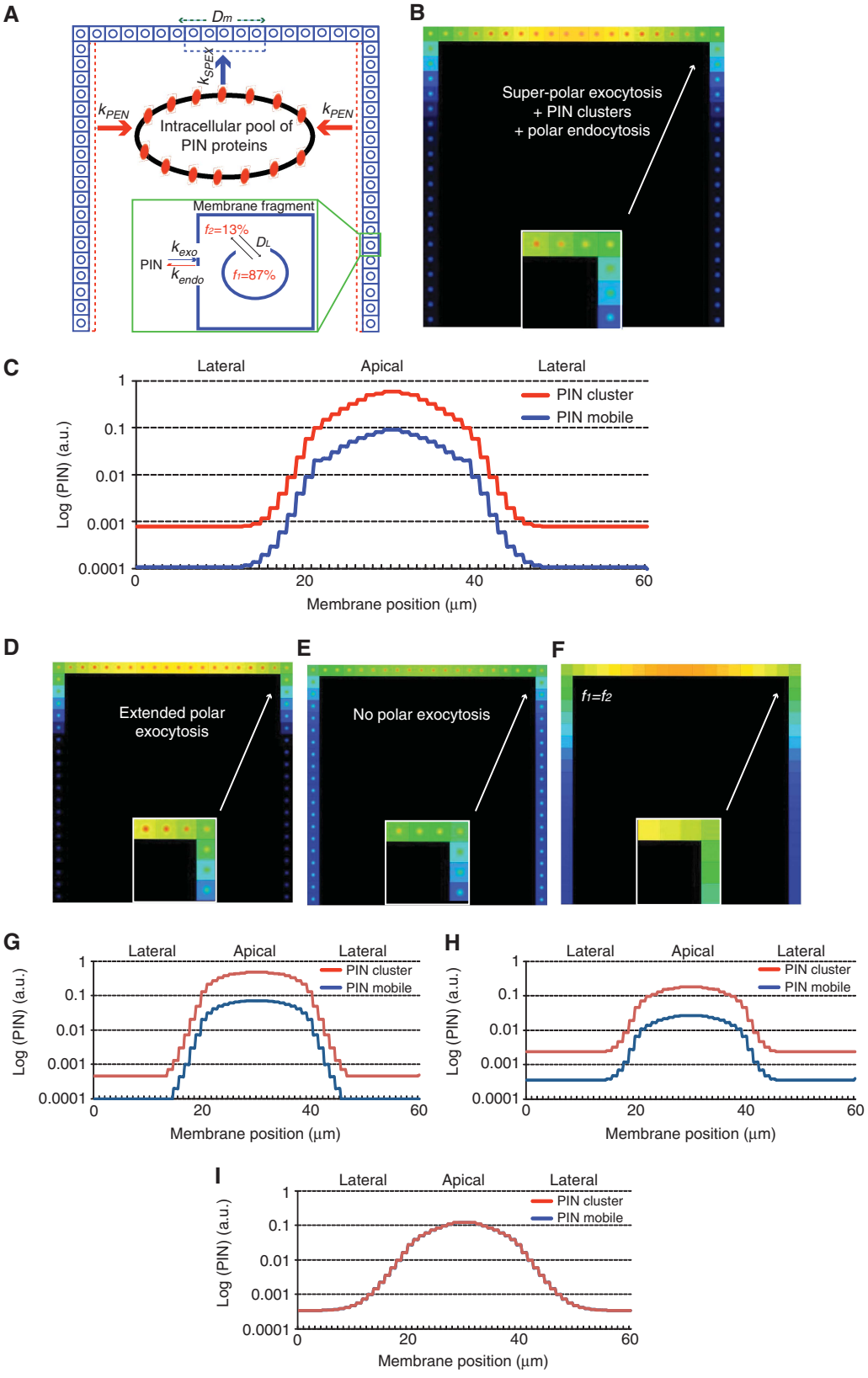
Next, we used our computer model to analyze whether polar PIN deposition reduced lateral PIN mobility and spatially defined PIN internalization at the lateral cell sides might together account for a robust PIN maintenance mechanism (Figure 6A).

Model simulations predicted the formation of a sharp PIN concentration gradient (bell-shaped distribution curve) in the plasma membrane (Figure 6B). The asymmetric distribution of PIN proteins within the plasma membrane was characterized by an increase of PIN proteins in the polar domain and an exponential decrease of PIN levels at the lateral surfaces of the cell (Figure 6B and C). The predicted decrease of PIN abundance at the lateral cell side was in agreement with our semi-quantitative confocal data ($\sim$1000-fold; Figures 6B, C, 1A and B). Our model predicted a 5-fold decrease of PIN levels from the center of the polar domain to its periphery (Figure 6B and C), which is in accordance with our experimental observations (Supplementary Figure 2). These findings illustrate that the computational model reproduces experimental observations (Figure 1A–C) and indicate that plant cell polarity could be maintained by a general mechanism integrating (i) super-polar delivery, (ii) reduced lateral diffusion in the plasma membrane, and (iii) spatially restricted internalization. We tested whether super-polar delivery of PIN to the polar domain is strictly required for PIN polarity maintenance in our model and allowed PIN exocytosis to the whole polar domain (Figure 6D). We found that the shape of the PIN gradient in the plasma membrane and the bell-shaped distribution curve characteristic of the PIN polarity (Figure 6G) were similar to those in the control simulations (Figure 6C) and experiments (Figure 1A–C). Next, we released the assumption of the polar PIN delivery in our model (for more details, see Supplementary information). By keeping endocytosis and exocytosis in balance, the model predicted an overall reduction of PIN levels in the plasma membrane and enhanced PIN labeling at the lateral surface of the cell (Figure 6E), but the preferential PIN polarity/asymmetry was preserved (Figure 6H). Interestingly, this finding was consistent with the experimental data that inhibition of the polar PIN recycling did not fully impair the preferential polar PIN localization (Figure 1G). Moreover, our model predictions were robust with respect to the model parameter manipulation in the biologically feasible range (Supplementary Figure 7).

These results demonstrate that super-polar delivery of PIN proteins might not be sufficient to maintain PIN polarity in plant cells, but, instead, that the role of super-polar PIN targeting could be in focusing or separating directional auxin fluxes within a given tissue. Also, our hypothesis might explain why not all epidermal cells show super-polar PIN localization (Figure 1C).

Finally, to address the importance of PIN clustering for PIN polarity maintenance, we gradually increased mobile (f_1=f_2) PIN fractions in our model (Figure 6F). The model predicted

Figure 6 Conceptual model for interweaving polarity mechanism. (**A**) The model combines super-polar delivery of PIN proteins (Figure 1H), PIN immobilization in membrane clusters (Figure 4A) and spatially defined PIN endocytosis (k_{SPEX}; thick red arrow) at the whole lateral sides of the cell. (**B**, **C**) Simulations of the combined model predicted a bell-shaped, graded pattern of the PIN distribution in the plasma membrane (B), similar to the *in planta* observations (Figure 1A–C and G; Supplementary Figure 2; see also Supplementary information and Supplementary Movie 3). (C) Steady-state PIN distribution profiles display a highly defined apical PIN polar domain in the cell. (**D**, **E**) Model alterations either by polar PIN deposition along the whole apical cell side (D) or by assuming non-polar PIN exocytosis (E) do not affect qualitatively PIN polarization (Supplementary information; Supplementary Movies 4 and 5). Although some minor effects on overall PIN distributions at cell edges were observed (D and E, white arrows). (**F**) Reduced PIN immobilization in membrane clusters (f_1=f_2) leads to enhanced PIN diffusion and ectopic PIN localization to the lateral cell side (Supplementary information; Supplementary Movie 6), as compared with non-altered model simulations (B). (**G**–**I**) Alterations in steady-state PIN distribution profiles for model simulations (D–F). Color coding scheme for simulations is as in Figure 1K.



the substantial leakage of PIN proteins to the lateral membrane domains (Figure 6F and I), similar to experimental observations (Figure 3F–H).

Both our experimental and *in silico* data suggest that, beside spatially defined PIN internalization (Figure 5), the reduced lateral PIN diffusion in the plasma membrane

is strictly necessary to maintain polar PIN domains in plant cells.

Discussion

Mechanisms of cellular polarization (Tepass *et al*, 2001; Lu and Bilder, 2005; Humbert *et al*, 2006; Wells *et al*, 2006; Munro and Bowerman, 2009; Chen *et al*, 2010) are remarkably well conserved in animal species (Shivas *et al*, 2010). Three central polarity complexes, such as Crumbs, Scribble, and PAR modules, localize to and specify a distinct subdomain of polarized cells (Shivas *et al*, 2010). These polarity modules are by far best described in epithelial cells of mammals, but also operate in non-epithelial cells such as in *C. elegans* embryos (Munro and Bowerman, 2009; Nelson and Beitel, 2009). Traditionally, polar delivery/exocytosis has drawn most attention for polarity establishment and maintenance (Altschuler *et al*, 2008). However, studies in yeast and epithelial cells highlighted the importance of reduced lateral diffusion for polarity maintenance (Valdez-Taubas and Pelham, 2003; Oh and Bi, 2011). The plasma membrane of epithelial cells is divided into apical and basolateral domains. Epithelial cells have tight cell-to-cell junctions (Giepmans and van Ijzendoorn, 2009) that are functional diffusion barriers and separate the apical and basolateral membranes from each other and have important roles in polarity maintenance (Wells *et al*, 2006). Recent growing evidence suggests that besides polar targeting and reduced lateral diffusion, the regulation of endocytosis is equally important for polarity maintenance in animal cells (Dudu *et al*, 2004; Shivas *et al*, 2010). However, the underlying mechanism of how endocytosis contributes to cell polarity still needs to be unraveled. In budding yeast, the regulation of CDC42p exocytosis and endocytosis and the ring-like, septin-based diffusion barriers (Oh and Bi, 2011) are similarly instructive for cell polarity maintenance (Orlando *et al*, 2011).

On the contrary to animal and yeast models, cell wall-encapsulated plant cells lack cell-to-cell junctions (Geldner, 2009) and ring-/septin-like diffusion barriers have been only reported in one particular cell type, namely the endodermis (Roppolo *et al*, 2011). Moreover, the prominent molecular components of animal polarity complexes are absent in plants (Geldner, 2009) and, hence, the molecular mechanisms underlying plant cell polarity are largely obscure. Previous work suggested that initial secretion of newly synthesized PIN proteins is non-polar, indicating that PIN endocytic recycling is crucial for establishment of polar deposition of PIN proteins at the specific cell side (Dhonukshe *et al*, 2008). Here, we extend these findings and address, by which mechanism PIN proteins maintain their polar localization within fluid membrane environment, once the polarity decision has been made. We propose a mechanism for dynamic PIN polarity maintenance in plants, which depends on an interweaving mechanism of (i) super-polar delivery to the center of the polar plasma membrane domain, (ii) recruitment to clusters in the plasma membrane that limits lateral cargo diffusion, and (iii) a spatially restricted polar endocytosis. Our results revealed that, similar to the generation of PIN polarity after non-polar secretion, the constitutive endocytic recycling has an important role also in the process of PIN polarity maintenance. However, in contrast to PIN polarity establishment, our *in silico* analysis suggests that not the super-polar PIN recycling, but the spatial regulation of PIN endocytosis and PIN protein immobilization in the plasma membrane is central to maintain PIN polarity.

Our findings indicate that filipin-sensitive PIN protein recruitment/exclusion to membrane clusters affects lateral mobility of PIN proteins, such as PIN1 and PIN2, and substantially contributes to PIN polarity maintenance.

Plant cells are not only competent to maintain but also to alter the polar PIN localization for complex tissue reprogramming—for instance during postembryonic organ formation (Benkova and Bielach, 2010). Therefore, reduced PIN diffusion due to plasma membrane compartmentalization might have not only clear functional importance for PIN polarity maintenance, but might also have been key in the evolution of dynamic cell polarity alterations. In plants, lateral diffusion within the plasma membrane appears to be limited by the cytoskeleton and the cell wall components (Feraru *et al*, 2011; Martiniere *et al*, 2011). Future work will address whether the non-mobile PIN clusters are eventually associated with the cytoskeleton or/and the cell wall.

Our study illustrates that plant cells have mechanisms to regulate polar PIN exocytosis (presumably by endosomal movement and spatially defined, short range vesicle trafficking), but also evolved a mechanism for spatially defined PIN endocytosis. Notably, distinct regions for exocytosis and endocytosis to and from the plasma membrane have been reported in animal and plant cells, such as following plant cytokinesis, tip growth in root hairs/pollen tubes and in animal neuronal synapses (Kidokoro, 2006; Takeda *et al*, 2008; Boutte *et al*, 2010; Zhao *et al*, 2010). Our findings further extend this view and are suggestive of an evolutionarily conserved mechanism of differentially active zones for exocytosis and endocytosis that would partially account for polarity maintenance.

Finally, our computer model simulations suggest that the partial loss of one of the PIN polarity components can be counteracted by the remaining ones, leading to PIN protein enrichment at its designated position, thereby safeguarding directional auxin transport. We assume that the multicomponent nature of the polar targeting mechanism proposed by the model may explain the difficulties in genetically interfering with PIN polarity or more generally with cell polarity, which remains a challenge for plant cell and developmentally biology (Grunewald and Friml, 2010).

Although this study largely focused on the maintenance of apical polar domain in epidermis, the principles of this model might be applied to any other polar domain that is manifest in plant cells.

Materials and methods

Plant material, growth conditions, and drug treatments

Plant material was *PIN2*::*PIN2-GFP* (Xu and Scheres, 2005), *PIN1*::*PIN1-GFP* (Benkova *et al*, 2003), *PIN2*::*PIN1-GFP-2;eir1-1* (Wiśniewska *et al*, 2006), *PIN2*::*PIN1-GFP-3* (Wiśniewska *et al*, 2006), *35S*::*GFP-PIP2a* (Cutler *et al*, 2000) and *35S*::*GFP-LTI6b* (Cutler *et al*, 2000).

Seedlings were grown vertically in Petri dishes on 0.8% agar 0.5 × Murashige and Skoog (MS) medium with 1% sucrose (pH 5.9) at 21°C under long day condition.

Immunolocalization

Immunolocalizations were performed on 5-day-old seedlings by using Intavis *in situ* pro robot according to the published protocol (Sauer *et al*, 2006). Primary antibody was rabbit anti-PIN2 (Abas *et al*, 2006) 1:2000 and anti-PIN1 (Paciorek *et al*, 2005) 1:2000. Secondary antibody was Cy3 anti-rabbit (Sigma-Aldrich) 1:600.

Quantification of the relative mobile fraction

Relative mobile fraction of PIN2–GFP was quantified by FRAP. A × 100 objective (1.4 NA) was used at a digital zoom setting of 4. Pre-bleaching and post-bleaching imaging was done using a 488-nm beam set at 50% output and 5% transmission. Three scans were made to establish the pre-bleach intensity and then a circular region of interest (ROI) of 5.5 μm^2 was drawn in a median optical section of the fluorescence plasma membrane. Fifteen iterations of 488 nm set at 100% transmission were used for bleaching. Recovery of the fluorescence was recorded during 131.7 s with a delay of 2.5 s between frames. Images were 256 × 256 pixels and were made with a scan speed of 0.493 s per frame. We confirmed that the energy of 488 laser used to record post-bleach data had no bleaching effect by recording region unbleached ROI. To assess potential differences of lateral PIN2 diffusion within the apical cell side, we have bleached either 2 μm diameter in center or in the periphery of the apical cell side. For FRAP analysis in stele cells (pPIN1:PIN1–GFP), we have used a × 40 objective; zoom 5; 512 × 512 images; scan speed 0.986 s; 22 scans for a total time of 102 s so around 5.96 s between each frames; ROI at 2 μm diameters; laser set at 21%. All other setting as for the first set of experiments. Obviously, the results cannot be as reliable as with a × 100 objective and bleaching was reduced by changing delay between frames. For analysis of the FRAP data to obtain the relative mobile fraction, we first normalized data by using the following equation: $I_n = [(I_t - I_{min})/(I_{max} - I_{min})] \times 100$; where I_n is the normalized intensity, I_t is the intensity at any time t, I_{min} is the minimum post-photobleaching intensity, and I_{max} is the mean pre-photobleaching intensity. Non-linear regression was used to model the normalized FRAP data. In this case, a two-phase exponential association equation was used: $Y(t) = A + B\,(1 + \exp^{(-K_1)(t)}) + C\,(1 - \exp^{(-K_2)(t)})$; where $Y(t)$ is normalized intensity, A, B, C, K_1, and K_2 are parameters of the curve, and t is time. Then, the value of their $Y(t=124\,s)$ was calculated and used as an approximation of the relative mobile fraction. In all, 17–23 cells from at least four different 7-day-old seedlings were analyzed. The seedlings were immobilized to prevent focus shift during scanning by mounting them in 1% low-melting point agarose cooled down to room temperature. The cover slip was sealed with VALAP (Vaseline:lanolin:paraffin wax).

Microscopy

For confocal laser scanning microscopy, a Leica TCS SP2 AOBS with upright microscope stand and an Olympus fluoview FV10 with inverted microscope stand were used. Semi-quantitative confocal imaging was performed and analyze with FV10. Images were processed in Adobe Photoshop CS2 and assembled in Adobe Illustrator CS2 (Adobe Inc.). Fluorescence signal intensity was analyzed with Image J 1.37v (Rasband) and confocal software (Leica). Data were statistically evaluated with Excel 2003 (Microsoft). The STED microscopy set-up was essentially as described previously (Hein *et al*, 2008). In short, Venus Fluorescence Protein was excited at 490 nm by a diffraction-limited spot, which was overlaid with a doughnut shaped STED beam of 590 nm. The STED focal doughnut was created by introducing a polymeric phase plate (RPC Photonics, Rochester, NY) applying imprinting a helical phase ramp of $\exp(i\varphi)$, with $0 < \varphi < 2\pi$, on the STED beam. The excitation and STED beams were overlapped by a dichroic mirror and then focused by a 1.3 NA objective lens (PL APO, × 63, glycerol, Leica, Germany). The epifluorescence was filtered with a 535/50 bandpass and detected by an avalanche photo diode. Images were recorded with resonant mirror scanning (15 kHz, SC-30; EOPC, Glendale, NY) along the x axis and stage scanning along the y axis (P-733, Physik Instrumente, Karlsruhe, Germany). For VAEM observation, 5-day-old Arabidopsis root epidermal cells expressing PIN2–GFP was subjected to vital imaging by using fluorescence microscope (Nikon Eclipse TE2000-E and CFI Apo TIRF 100XH/1.49 numerical aperture objective) equipped with Nikon TIRF2 system. PIN2–GFP was excited at 488 nm Argon laser. In VAEM, each frame was exposed for 100 ms. Image was acquired with an Andor iXonEM EMCCD camera.

Computational methods

All model simulations were performed until a steady state emerged (on the average time scale of 3 h (CPU)). The simulations were done by numerical computations of coupled ODE systems, with an adaptive-size, fifth-order Runge-Kutta method. All figures were processed in Adobe Illustrator. Figures 1J, 4B, 6B and D–F, Supplementary Figures 5 and 7 and Supplementary Movies 1–6 are screenshots from model simulations. For full details of the computer model, we refer to Supplementary information.

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (www.nature.com/msb).

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Author contributions: JK-V, KW, and JF conceived the study and designed the experiments. JK-V, KW, AM, HT, KW, SN, JL, SJ, SR, CL, WG, SH, JR, and JF carried out the experiments and analyzed the data. JK-V, KW, and JF wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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