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Chapter 3

PIN phosphorylation by AGC kinases enhances plasma membrane abundance of these auxin efflux carriers by reducing vacuolar targeting

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Abstract

The plant hormone auxin regulates a plethora of developmental programs through polar auxin transport (PAT) generated maxima and minima. Key drivers of this cell to cell transport are the PIN FORMED (PIN) efflux carriers that determine the direction of auxin flow through their asymmetric distribution at the plasma membrane (PM). PIN abundance and polar localization are regulated by dynamic endocytosis and recycling to the PM, or alternatively, by targeting PINs to the vacuole for degradation. PIN vacuolar targeting is important for the regulation of root gravitropism and negative phototropism. The *Arabidopsis* AGC3 protein kinases PINOID (PID), WAG1 and WAG2 have been shown to direct polar PIN PM distribution by phosphorylating serine residues located in three conserved TPRXS(N/S) motifs in the PIN central hydrophilic loop (HL). Here we show, using fluorescent protein-tagged PIN1 and PIN2 versions with non-phosphorylatable or phosphomimic mutations, that phosphorylation by AGC3 kinases reduces PIN vacuolar targeting in protoplasts, embryos and roots. The effect of phosphorylation is dose-dependent, as complete loss-of-phosphorylation leads to a significantly stronger enhancement of PIN vacuolar targeting compared to single or double serine to alanine substitutions. Our data point to a dual function for AGC3 kinase-mediated PIN phosphorylation, which besides PIN polarity also controls the abundance of these auxin efflux carriers at the PM of plant cells.

Introduction

Plant development and growth are regulated through the directional cell-to-cell transport of the plant hormone auxin. This polar auxin transport (PAT) is mediated for a large part by PIN-FORMED (PIN) auxin efflux carriers. In the model plant *Arabidopsis thaliana*, the PIN family is composed of eight members, of which the five PIN1-type or long PIN proteins (PIN1, PIN2, PIN3, PIN4 and PIN7) are now recognized as drivers of PAT. Jointly they determine the direction of auxin flow through their asymmetric distribution at the plasma membrane (PM) (Mravec et al., 2009; Petrásek et al., 2006; Wisniewska, 2006). Polar PIN localization is established by dynamic subcellular trafficking processes that are modulated by auxin itself (Paciorek et al., 2005; Sauer et al., 2006), developmental signals (Benková et al., 2003; Friml et al., 2003), and by environmental cues (Ding et al., 2011; Friml et al., 2002; Harrison and Masson, 2008; Kleine-vehn et al., 2010).

The subcellular trafficking of long PIN proteins has been most extensively investigated for *Arabidopsis* PIN1 and PIN2. These studies have led to the model that following the placement of *de novo* PIN proteins at the PM, polarity is established and maintained by constitutive cycling of PIN cargoes between the PM and endosomes (Dhonukshe et al., 2007; Geldner et al., 2003). Concurrently, overall PIN abundance at the PM is adjusted by redirecting endosomes or multi-vesicular bodies (MVB's) to the lytic vacuole for degradation of their PIN cargo. Vacuolar targeting of PIN2 was found to be important during the root gravitropic and phototropic responses and to involve 26S proteasome activity and auxin- and light signaling-induced PIN ubiquitination (Abas et al., 2006; Baster et al., 2012; Laxmi et al., 2008; Wan et al., 2012; Zhang et al., 2013). PINs sorting to a multi-vesicular body (MVB) happens through the action of the ESCRT homolog CHARGED MULTIVESICULAR BODY PROTEIN/CHROMATIN MODIFYING PROTEIN1 (CHMP1) (Spitzer et al., 2009) and the de-ubiquinating protein ASSOCIATED MOLECULE WITH THE SH3 DOMAIN OF STAM3 (ASMH3) (Isono et al., 2010). From this point there is the possibility of PINs moving back from the TGN/EE to the plasma membrane through the action of the retromer, consisting of among others SORTING NEXIN 1 (SNX1), VACUOLAR PROTEIN SORTING 29 (VPS29) and VPS35 (Jaillais et al., 2006; Jaillais et al., 2007; Kleine-Vehn et al., 2008b). PIN trafficking from the PM to endosomes involves the ARF GTPase activating protein (GAP) protein VASCULAR NETWORK 3/SCARFACE (VAN3/SFC) (Koizumi et al., 2005) (Koizumi et al., 2005; Sieburth et al., 2006), whereas the brefeldin A (BFA)-sensitive ADP-ribosylation factor guanine nucleotide exchange factor (ARF GEF) GNOM mediates polar recycling from endosomes to the PM (Geldner et al., 2003; Steinmann, 1999). Short-term high dose BFA treatment induces reversible intracellular accumulation of PIN1 protein in so called BFA compartments (Geldner et al., 2001). BFA was also found to inhibit PIN vacuolar targeting, and experiments with plants expressing a BFA resistant version of GNOM suggest that this is mediated by another BFA-sensitive ARF-GEF (Kleine-Vehn et al.,

2008b). Apically (shootward) localized PINs are less sensitive to BFA, indicating that apical recycling is mediated by a BFA-insensitive ARF-GEF. In line with this conclusion, long-term treatment with lower BFA concentrations induces transcytosis of basally (rootward) localized PINs to the apical domain (Kleine-Vehn et al., 2008a).

The same basal-to-apical polarity switch can be achieved by the activity of three redundantly acting AGC3 protein serine/threonine kinases PINOID (PID), WAG1 and WAG2. In root cells, overexpression of these AGC3 kinases induces a basal-to-apical PIN polarity switch and a depletion of the organizing auxin maximum in the primary root meristem, subsequently leading to agravitropic root growth and meristem collapse. In contrast, in the embryo and the inflorescence meristem, loss of this kinase activity causes an apical-to-basal PIN1 polarity shift, resulting in pin-like inflorescences and embryos with aberrant cotyledon numbers or no cotyledons (Dhonukshe et al., 2010; Friml et al., 2004). It has been shown that the three AGC3 kinases direct PIN polarity by phosphorylating the serine residue of three conserved TPRXS motifs present in the large central hydrophilic loop (HL) of long PIN proteins (Dhonukshe et al., 2010; Huang et al., 2010). AGC3 kinases act antagonistically with PP2A/PP6-like heterotrimeric phosphatases that dephosphorylate the PIN-HL (Friml et al., 2004; Huang et al., 2010). Further analysis showed that phosphorylation of the PIN-HL recruits PIN proteins from the basal recycling pathway to the GNOM-independent apical recycling pathway (Kleine-Vehn et al., 2009).

Earlier studies already suggested that the PID kinase, besides its role as a PIN polarity determinant, is also a positive regulator of PAT (Benjamins et al., 2001; Lee and Cho, 2006). Here we confirm this notion by showing that phosphorylation by PID enhances PIN PM abundance and that in the absence of phosphorylation PIN proteins show an enhanced relocation from the PM to the lytic vacuole. Partial phosphorylation of PINs gradually decreases their PM polarity. AGC3 kinases WAG1, WAG2 and AGC3-4 and the AGC1-type D6 protein kinases act in a similar manner to PID and can decrease vacuolar targeting of PIN1, although this seems to be at least partially independent of TPRXS phosphorylation.

Results

PID activity enhances auxin efflux in tobacco BY-2 cells

To find direct evidence for the positive effect of PID on auxin efflux, the tamoxifen inducible *pINTAM>>PID* was stably introduced into tobacco BY-2 cells or in a BY-2 cell line expressing *PIN1::PIN1-GFP*. Semi-quantitative RT-PCR analysis showed that PID expression was induced following tamoxifen treatment (Fig. 1A). In the *pINTAM>>PID + PIN1::PIN1-GFP* transgenic cell line PIN1-GFP was well-expressed and localized predominantly at the PM (Fig. 1B). Without the induction of *PID* the GFP signal strongly internalized in some cell

files, most likely caused by vacuolar targeting of PIN proteins (not shown). In untreated cells ^3H -labelled NAA accumulated to 1 pmol per million cells, whereas tamoxifen-induced *PID* expression resulted in a 30% reduction of the ^3H -NAA accumulation, indicating that *PID* enhances auxin efflux (Fig. 1C). This result corroborates the previously suggested role for *PID* as positive regulator of auxin efflux (Benjamins et al., 2001; Lee and Cho, 2006), and suggests that *PID*-dependent phosphorylation of PIN proteins stimulates their PM localization.

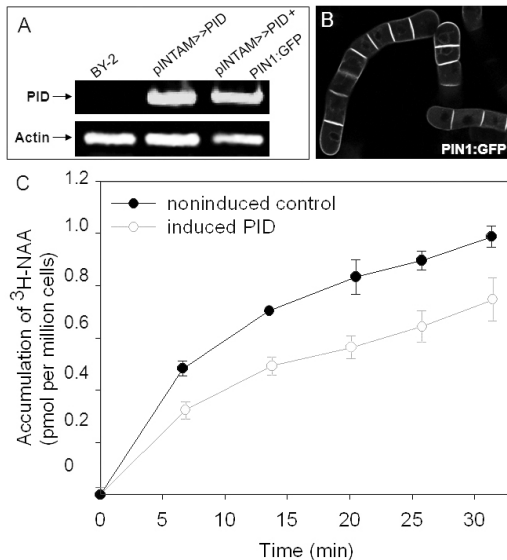


Figure 1: *PID* expression decreases auxin accumulation in BY-2 cells.

(A) Semi quantitative RT-PCR analysis detects *PID* expression in the transgenic *pINTAM>>PID* or *pINTAM>>PID + PIN1-GFP* BY-2 cell lines after 48 hours induction with 5 μM tamoxifen, but not in BY-2 cells. (B) PIN1-GFP localization in *pINTAM>>PID + PIN1-GFP* BY-2 cells. (C) Accumulation of ^3H -NAA (2 nM) in *pINTAM>>PID + PIN1-GFP* BY-2 cells after 48 hours treatment with DMSO (non-induced control) or 5 μM tamoxifen (induced PID).

***PID*-dependent phosphorylation reduces PIN1 vacuolar targeting in protoplasts**

To obtain more mechanistic insights into the effect of *PID*-mediated PIN phosphorylation on PAT, we used an *Arabidopsis* suspension-based protoplast system to study PIN turnover. Following transfection and before observation these protoplasts were standardly incubated overnight in the dark, a procedure known to stabilize GFP in the lytic vacuole (Tamura et al., 2003). In protoplasts transfected with *35S::PIN1-GFP* and incubated for 17 hours in the dark, PIN1-GFP could be observed at the PM and at intracellular structures (Fig. 2A), consistent with previous observations (Dhonukshe et al., 2007). In 50-60% of the protoplasts the GFP signal also accumulated in vacuole-like structures (Fig. 2B,C). Interestingly, prolonged incubation for 19, 21 and 23 hours after transfection resulted in a gradual increase of the percentage of protoplasts showing a vacuolar GFP signal (Fig. 2G). This suggests that in protoplasts PIN1-GFP becomes increasingly targeted to the vacuole following its initial appearance at the PM. These results indicate that the protoplast system is a convenient and efficient test system to study the effect of AGC kinases on PIN1 PM abundance and turnover.

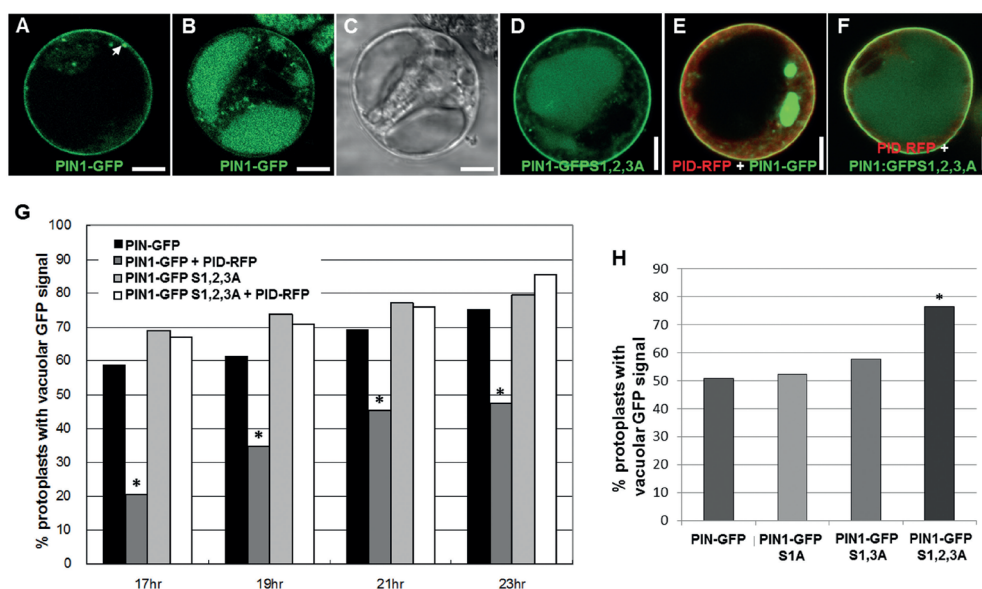


Figure 2: Phosphorylation-dependent control of PIN1 vacuolar targeting in *Arabidopsis* protoplasts.

(A-F) Confocal images of representative transfected *Arabidopsis* protoplasts. (A) Protoplast expressing PIN1-GFP, showing GFP signal at the PM and at endosomal punctuate structures (arrow). (B) PIN1-GFP expressing protoplast, where GFP signal accumulates in the vacuole. (C) Bright field image of the protoplast in (B), showing the location of the two vacuoles. (D) Protoplast expressing PIN1-GFP S1,2,3A, showing predominant vacuolar GFP signal. (E) PID-RFP co-transfection (PID can be found mainly at the PM) decreases vacuolar targeting of PIN1-GFP, while it does not with PIN1-GFP S1,2,3A (F). (G) *Arabidopsis* protoplasts were transfected with PIN1-GFP (black), PIN1-GFP S1,2,3A (light gray), PIN1-GFP and PID-RFP (dark gray), or PIN1-GFP S1,2,3A and PID-RFP (white). Protoplasts were observed and counted after incubation in darkness for 17, 19, 21 or 23 hrs. (asterisks: chi-square test of independence comparing ratios between transfection with or without PID-RFP, $p < 0.01$). (H) *Arabidopsis* protoplasts were co-transfected with PID-RFP and PIN1-GFP, PIN1-GFP S1A, PIN1-GFP S1,3A or PIN1-GFP S1,2,3A, and were imaged and counted after incubation in darkness for 19 hrs (asterisk, chi square test, $p < 0.01$).

Enhanced vacuolar accumulation was observed when the loss-of-phosphorylation *35S::PIN1-GFP S1,2,3A* mutant construct encoding the PIN1-GFP S1,2,3A fusion protein with all three Serines in the conserved TPRXS motifs substituted by Alanines, was transfected (Fig. 2D and G). Enhanced phosphorylation of PIN1 in protoplasts co-transfected with *35S::PIN1-GFP* and *35S::PID-mRFP* (hereafter referred to as *PID-RFP*) resulted in a significant reduction of the number of protoplasts showing vacuolar accumulation of GFP (Fig. 2E and F). Transfection of *35S::PIN1-GFP S1,2,3A* in combination with *PID-RFP* did not significantly alter the vacuolar accumulation (Fig. 2F and G). Independent experiments with the protoplast system showed that the same results could be reproducibly obtained (Fig. 7A).

Dose-dependent effect of phosphorylation on PIN1 vacuolar targeting and polarity

When protoplasts were transfected with wild type *35S::PIN1-GFP*, or with mutant versions coding for fusion proteins where one, two or three of the serines in the conserved TPRXS motifs were substituted by an alanine, a significant increase in vacuolar targeting was observed for the PIN1-GFP S1,2,3A variant, but not for the other two mutant variants compared to the wild-type control (Fig. 2H). These results suggest that there is a dose-dependent effect of phosphorylation on vacuolar targeting, and that partial phosphorylation is sufficient to reduce vacuolar targeting.

To corroborate these results *in planta*, we used *Arabidopsis pin1* mutants either containing the complementing wild-type *PIN1::PIN1-GFP* (hereafter referred to as *PIN1-GFP*), or the mutant *PIN1-GFP S1,2,3A* construct (Huang et al., 2010). In root stele cells of light-grown *PIN1-GFP* seedlings at five days post germination (dpg), the GFP signal was predominantly detected at the PM. Only limited intracellular signal was detected in *PIN1-GFP* roots, whereas this signal was slightly enhanced in *PIN1-GFP S1,2,3A* roots (Figures 3A and 3C). One hour dark treatment resulted in GFP accumulation in vacuoles for both *PIN1-GFP* and *PIN1-GFP S1,2,3A* seedling roots (Figures 3B and 3D), however, the vacuolar GFP signal in *PIN1-GFP S1,2,3A* roots (Fig. 3D) was much stronger than that in *PIN1-GFP* roots (Fig. 3B). Longer (24 hours) incubation of seedlings in darkness led to loss of PM signal for *PIN1-GFP S1,2,3A*, whereas *PIN1-GFP* retained some PM signal (Fig. 3E,F). These results are in line with the protoplast experiments, and indicate that also *in planta* vacuolar targeting of the phosphomutant *PIN1-GFP S1,2,3A* is enhanced compared to that of wild-type *PIN1-GFP*.

To look at the effect of *PID* overexpression on *PIN1-GFP (S1,2,3A)* localization, seeds of *PIN1-GFP (S1,2,3A)* lines in wild type or *35S::PID* background were germinated and grown on medium containing 0,3 μ M NPA, to prevent root meristem collapse in the *PID* overexpression background (Benjamins et al., 2001). After 1 hour of incubation in darkness, GFP signal could be observed in vacuolar structures in root stele cells of both *PIN1-GFP* and phosphomutant *PIN1-GFP S1,2,3A* seedlings, but the PM signal was stronger in *PIN1-GFP* roots (Fig. 3G and H). *PID* overexpression resulted in an increase in *PIN1-GFP* signal at the PM and caused *PIN1-GFP* to accumulate into internal compartments that are visually different from the vacuolar compartments (for example, Fig. 3G and H). *PID* overexpression had limited to no effect on the location of the *PIN1-GFP S1,2,3A* phosphomutant (Fig. 3I and J). These results confirm the protoplast experiments and indicate that *PID*-dependent phosphorylation at the TPRXS motifs decelerates vacuolar targeting of *PIN1-GFP*.

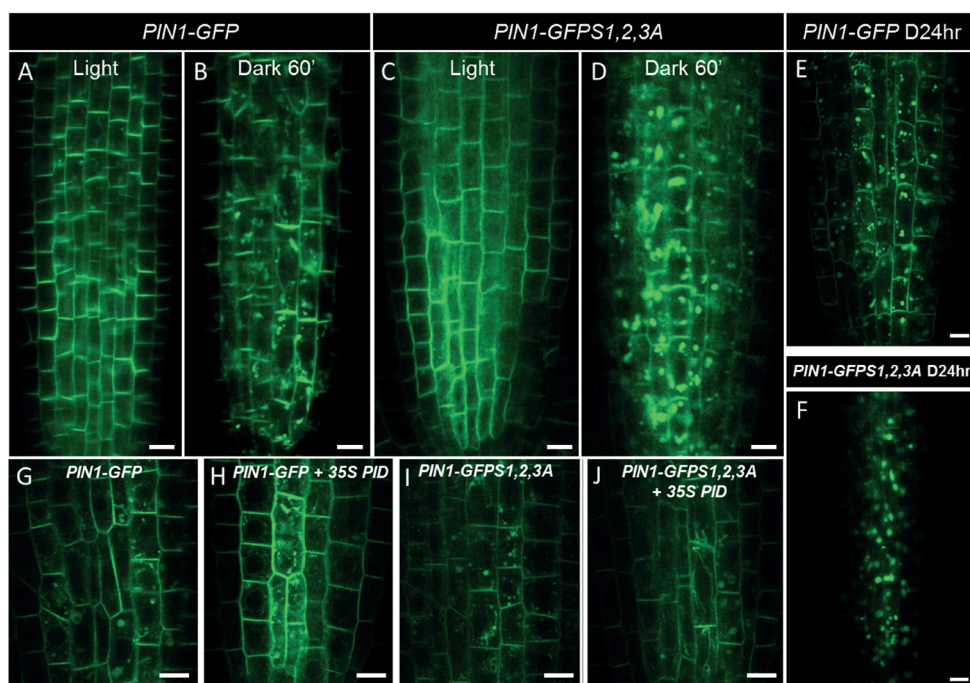


Figure 3: PIN1 phosphorylation reduces vacuolar targeting and increases PM abundance in *Arabidopsis* root stele cells.

(A-F) Confocal images of four day old *pin1-1* *Arabidopsis* seedling root stele cells expressing PIN1-GFP or PIN1-GFP S1,2,3A following incubation in light (A, C) or dark for 60 minutes (B, D) or dark for 24 hours (E, F). (G-J) Confocal images of root stele cells of four day old seedlings expressing PIN1-GFP (G, H) or PIN1-GFP S1,2,3A (I, J) crossed through the 35S::PID line 21 (H, J) germinated on 0,3μM NPA and incubated in the dark for 3 hrs.

To investigate the effect of partial PIN1HL phosphorylation, we observed embryos and seedling roots of *pin1* mutant plants expressing wild-type and different loss-of-phosphorylation variants of the PIN1-GFP fusion construct. Vacuolar targeting of PIN1-GFP occurred more readily in embryo protoderm cells than in root stele cells. In PIN1-GFP embryos, clear GFP positive vacuolar structures could already be observed after 30 minutes in the dark, while these were mostly absent in 1 hour dark incubated root stele cells, or in embryos directly observed after isolation (Fig. 4A, B first panels). PIN1-GFP S1A, PIN1-GFP S1,3A and PIN1-GFP S1,2,3A embryos displayed progressively intense vacuolar targeting even without dark treatment. For PIN1-GFP S1,2,3A, strong vacuolar targeting coincided with complete loss of PIN1 polarity (Fig. 4A, B). Also in PIN1-GFP S1,3A embryos clear PIN vacuolar targeting and reduced PIN polarity were observed, whereas in PIN1-GFP S1A embryos enhanced PIN vacuolar targeting was observed, while PIN1 polarity was maintained (pink arrows). In seedling roots, a slight increase in PIN1-GFP internalization and possible vacuolar signals could be observed in the PIN1-GFP S1A, -S1,3A and -S1,2,3A plants (Fig. 4C, white arrows). Only PIN1-GFP S1,2,3A showed complete loss of polarity,

whereas the other two mutant and the wild-type PIN1-GFP versions still showed predominant basal polarity (Fig. 4C, pink arrows). The combined results show that also *in planta* there is a dose-dependent effect of the phosphorylation status on PIN1 polarity and PM abundance. Complete loss-of-phosphorylation leads to both loss of polarity, enhanced internalization and vacuolar targeting in all cell types, whereas the effects of partial loss-of-phosphorylation seem to be more cell-type specific.

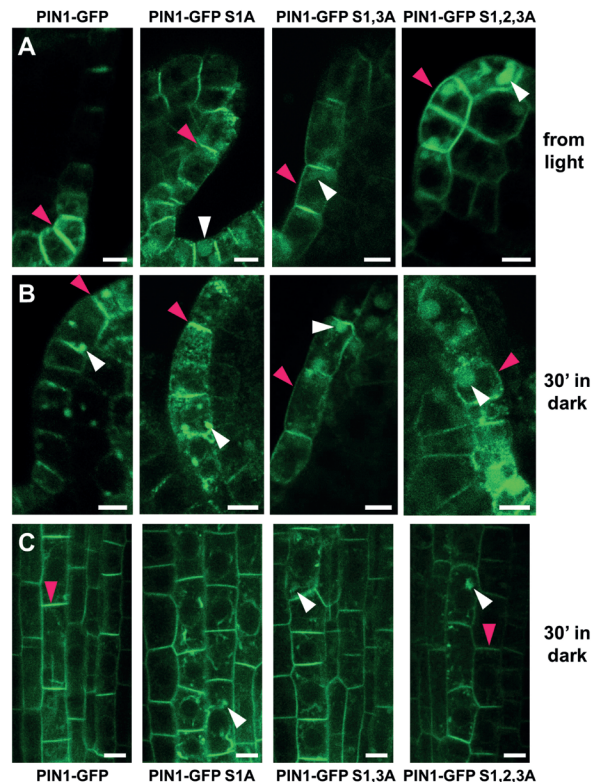


Figure 4: Dose-dependent effect of phosphorylation on PIN vacuolar targeting and -polarity in *Arabidopsis* embryos and roots.

(A,B) Confocal images of *Arabidopsis pin1-1* mutant embryo epidermis cells (late heart stage) incubated in light (A) or 30 minutes in darkness (B). (C) Four day old seedling root stele cells expressing *PIN1-GFP*, *PIN1-GFPS1A*, *PIN1-GFPS1,3A* or *PIN1-GFPS1,2,3A* incubated in darkness for 30 minutes. White arrowheads indicate vacuolar signal. Pink arrowheads indicate PIN1-GFP (a)polarity.

Phosphorylation enhances PIN2 PM abundance

To see if phosphorylation had a similar effect on the turnover of other PINs, we observed and quantified the subcellular localization of the YFP signal in *eir1-1 PIN2::PIN2-YFP* (*PIN2-YFP*) and *eir1-1 PIN2::PIN2-YFP S1,2,3A* (*PIN2-YFP S1,2,3A*) seedling roots (Dhonukshe et al., 2010). PIN2-YFP predominantly localized at the apical PM in epidermis cells of the root

meristem when seedlings were grown in light (Fig. 5A, and E). For PIN2-YFP S1,2,3A, the polarity was lost while the ratio of vacuolar over PM signal was increased compared to PIN2-YFP (Fig. 5C, E and F). Dark incubation further increased the ratio of vacuolar over PM signal for both proteins, but the increase was significantly higher for PIN2-YFP S1,2,3A as compared to wild-type PIN2-YFP (Fig. 5B,D-F). Because of the different pH sensitivity of the YFP (VENUS) protein compared to GFP (Nagai et al., 2002), a longer (5 hours) dark incubation was required to obtain sufficient vacuolar YFP signal. Interestingly, besides enhancing the vacuolar signal, the incubation in darkness also decreased the apical (shootward) polarity of PIN2-YFP (Fig. 5F), an effect that has not been reported previously. These results show that AGC3 kinase-mediated phosphorylation is also required for PIN2 PM abundance and polarity.

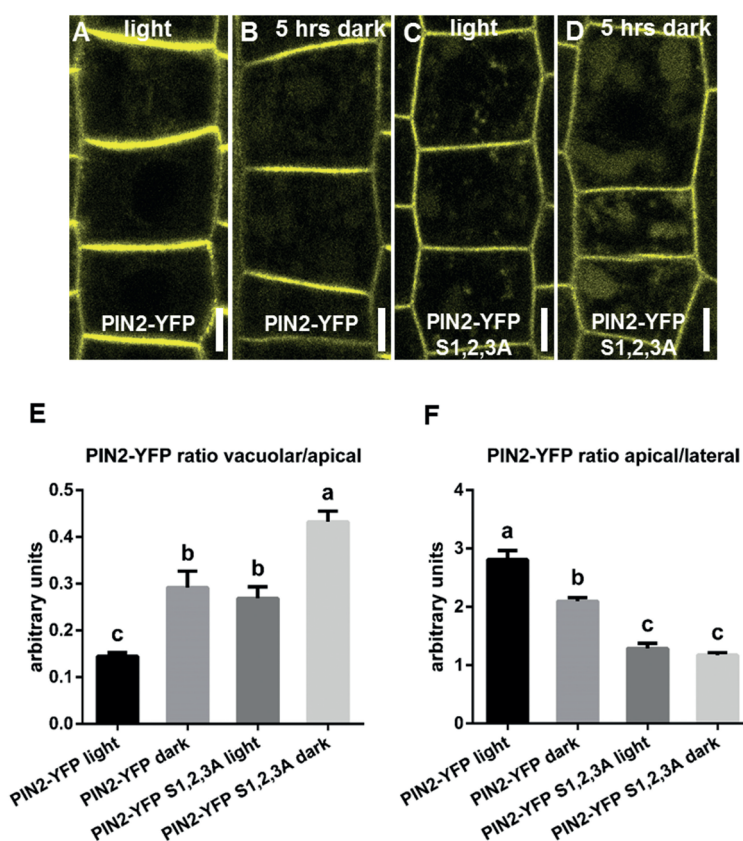


Figure 5: PIN2 phosphorylation reduces vacuolar targeting and increases polar localization in *Arabidopsis* root epidermis cells.

(A-D) Confocal images of root tip epidermis cells of 4 day old *PIN2::PIN2-YFP* (A,B) or *PIN2::PIN2-YFP S1,2,3A* (C,D) seedlings incubated in light (A,C) or for 5 hrs in darkness (B,D). (E) Quantification of the ratio of vacuolar and apical PM YFP signal of images as in (A-D). (F) Quantification displaying the ratio of the apical and lateral PIN2-YFP signal of images as in (A-D). Error bars denote the standard error of the mean, letters show statistical significant difference, one way ANOVA with post-hoc t-test $p < 0.05$.

PID phosphorylation enhances PIN1 abundance in membrane fractions

Co-transfection of PIN1-GFP with PID resulted in accumulation of the GFP signal at the PM and in endosomal structures (Fig. 2E, 3I and 6A). The size of the endosomal structures suggests that they can be MVB's (Tse, 2004). Protoplasts transfected with 35S::PIN1-GFP or 35S::PIN1-GFP S1,2,3A in the presence or absence of 35S::PID:FLAG and 1 μ M IAA were lysed and membrane fractions were analyzed on Western blot with anti-PIN1 and anti-GFP antibodies (Fig. 6B and Fig. S1). The ratio of PIN1-GFP to endogenous PIN1 was estimated to be 50:1 (Fig. S2) and therefore the band visible with the PIN1 antibody in Fig. 6B is that of PIN1-GFP. Co-transfection of PID increased the amount of PIN1-GFP in the membrane fraction, whereas auxin addition alone did not have a significant effect (Fig. 6B,C). Together with PID expression however, auxin addition resulted in an even higher PIN1 abundance in the membrane fraction (Fig. 6B,C). The effect of auxin is possibly due to an inhibition of endocytosis (Paciorek et al., 2005). Interestingly, PID co-transfection resulted in enhanced phosphorylation of both PIN1-GFP and PIN1-GFP S1,2,3A, as indicated by a higher weight smear on the Western blots (Michniewicz et al., 2007). Quantification and plotting of the results in an intensity graph showed that PIN1-GFP has an extra higher band smear than that of PIN1-GFP S1,2,3A (red arrow, Fig. 6D). The lower smear is also observed for the PIN1-GFP S1,2,3A mutant version, meaning that the lower smear is probably phosphorylation of non-TPRXS motifs. More detailed analysis using 40 or 60 μ M phostag to retard the motility of phosphorylated proteins confirmed that both the upper and lower smear are indeed due to phosphorylation of PIN1-GFP (Fig. 6E). The upper smear that was clearly visible on SDS-PAGE without phostag ran progressively slower through both concentrations used. Interestingly the difference between PIN1-GFP and PIN1-GFP S1,2,3A was relatively small (Fig. 6E red arrow). Phostag treatment resulted in clear separation of the lower smear from the band representing the unphosphorylated PIN1-GFP (Fig. 6E, black arrows). The fact that this band is also visible in the absence of PID overexpression or with the PIN1-GFP S1,2,3A version confirms our notion that PIN1-GFP is phosphorylated by PID in *Arabidopsis* protoplasts at one or more other sites than the TPRXS motif serines. This is in line with previously published in vitro phosphorylation results where the S1,2,3A mutant versions of the PIN1HL still showed a low level of phosphorylation (Huang et al., 2010). Here we show that phosphorylation at these unknown residues increases the amount of PIN1 found in membrane fractions, but does not influence the PID-mediated inhibition of vacuolar targeting (Fig. 6A). In the presence of PID both PIN1-GFP and PIN1-GFP S1,2,3A accumulate in endosomal compartments (Fig. 7B), suggesting that phosphorylation of these other sites affects PIN1 endosomal trafficking or recycling.

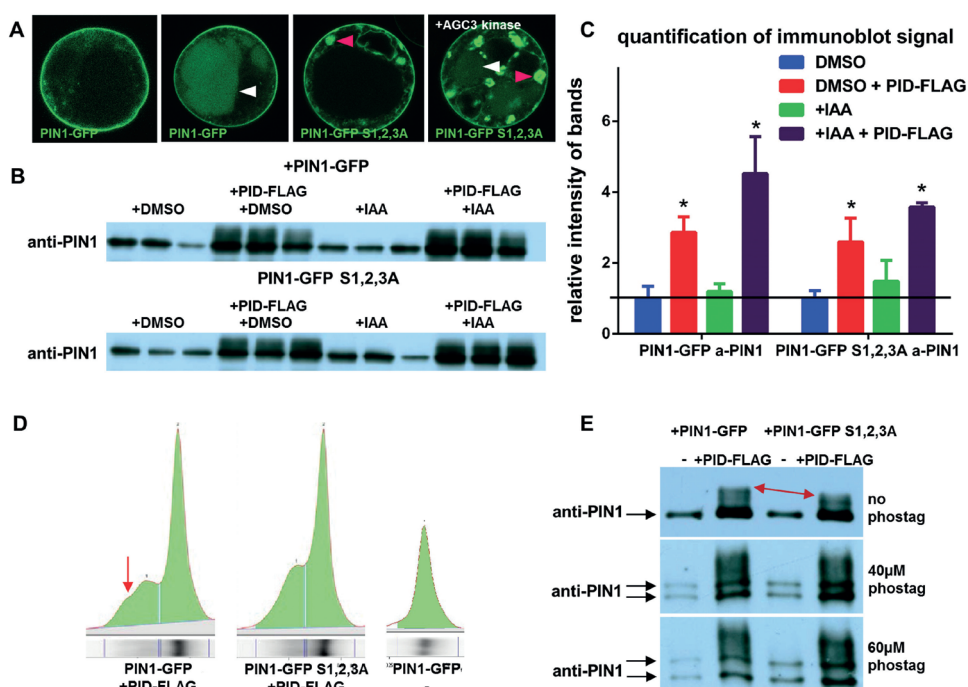


Figure 6: AGC3 kinase-dependent PIN1 phosphorylation enhances PIN1 abundance in plasma membrane and endomembrane fractions.

(A) Representative examples of the subcellular localization of PIN1-GFP or PIN1-GFP S1,2,3A expressed in *Arabidopsis* protoplasts without or with AGC3 kinases. White arrowheads highlight vacuolar signal, pink arrow heads depict strong accumulation in endosomal compartments, possibly MVB's. (B) Western blots of membrane fractions of *Arabidopsis* protoplasts expressing PIN1-GFP (upper panel) or PIN1-GFP S1,2,3A (lower panel) with or without PID-FLAG, and in the presence or absence of 1μM IAA. All transfections were performed in triplo and blots were probed with anti-PIN1 antibodies. (C) Quantification of signal from samples of (B), normalized to '+DMSO' samples (error bars show standard deviation in the three replicate samples, asterisks indicate a significant difference with control (DMSO), *t*-test, *p*<0,05). (D) Detailed analysis of Western blots in (B) shows that there is an extra peak of signal in the PIN1-GFP samples (red arrow) that is missing in the PIN1-GFP S1,2,3A samples. (E) PIN1-GFP (S1,2,3A) with or without PID-FLAG cotransfection run on SDS gel containing phostag (40 or 60 μM). Without PID co-transfection a separate band is visible due to phostag which is thus likely already phosphorylated (black arrows). PID-cotransfection caused additional higher bands while the difference between PIN1-GFP and PIN1-GFP S1,2,3A is one additional higher band (red arrow).

TPRXS phosphorylation-independent effect of AGC kinases on PIN1 trafficking

Besides PID, also its closest homologs the AGC3 kinases WAG1 and WAG2 have been shown to phosphorylate the PIN HL at the TPRXS motifs, leading to apical PIN localization. In case of the PIN2 HL no indication was found that other residues were phosphorylated by the AGC3 kinases (Dhonukshe et al., 2010). For the AGC1-clade kinase D6PK and its close homologs D6PKL1, 2 and 3 it has been shown that they can phosphorylate the PIN HL at different (non-TPRXS) residues S4 and S5 (Willige et al., 2013; Zourelidou et al., 2009;

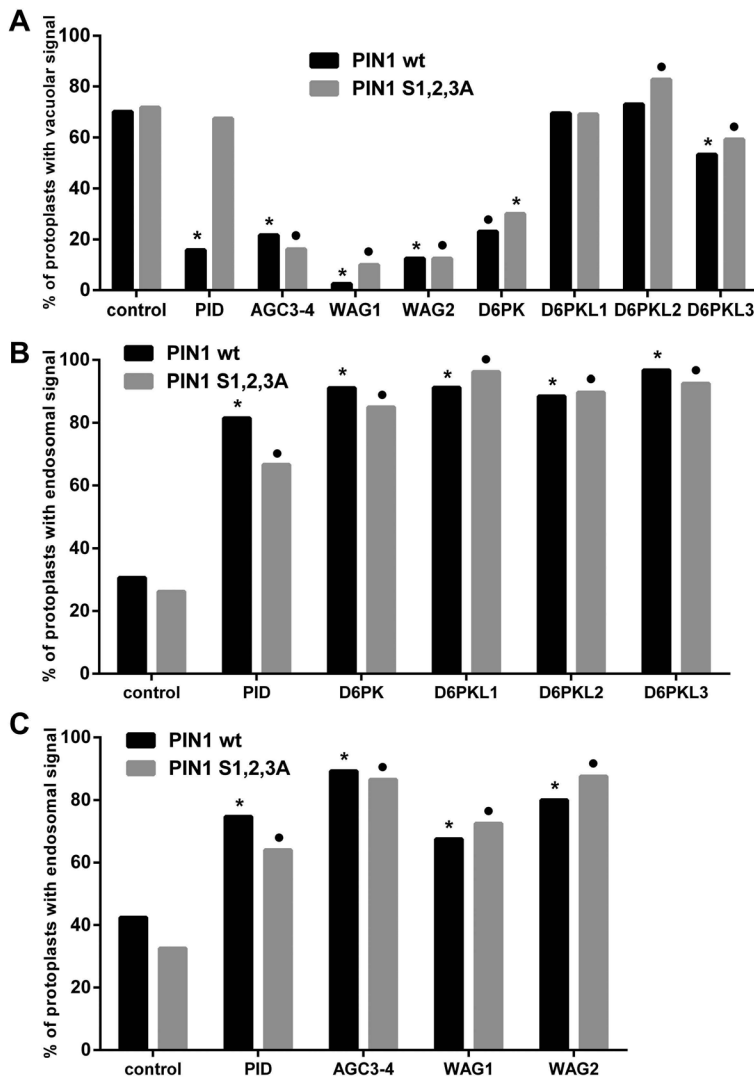


Figure 7: AGC3's and selected D6PK's can influence vacuolar targeting and endosomal accumulation of PIN1-GFP and PIN1-GFP S1,2,3A.

(A) Vacuolar targeting assay performed in a similar fashion to Fig. 2G, here with co-transfection of 35S driven mRFP fusion constructs of all the AGC3 kinases (PID, AGC3-4, WAG1, WAG2) and the AGC1-type D6PK's (D6PK, D6PKL1, D6PKL2, D6PKL3). PIN1-GFP (S1,2,3A) and AGC kinase expressing protoplasts were counted approximately 20 hours after transfection. (B, C) Protoplasts treated the same as in (A) were scored for the presence of strong endosomal accumulations (as can be seen in Fig. 6A (pink arrows)). The D6PK's (B) and the AGC3 kinases (C) all increase the percentage of cells with strong endosomal accumulations. (Asterisk and black dot indicate a significant difference ($p < 0.01$) in a chi-square test compared to the PIN1-GFP (PIN1 wt) or PIN1-GFP S1,2,3A (PIN1 S1,2,3A) controls, respectively.)

Zourelidou et al., 2014). Based on the analysis of mutant PIN versions, *Xenopus* oocyte efflux experiments and phosphorylation specific antibodies the authors concluded that PID- and D6PK-mediated phosphorylation of S4 and S5 is important for the activation of PINs (Weller et al., 2017; Zourelidou et al., 2014). Here, we tested the effect of the D6 kinases on vacuolar targeting and endosomal accumulation in protoplasts (Fig. 7). Also in this experiment, PID phosphorylation of the TPRXS motif serines was essential to reduce vacuolar targeting of PIN1. Surprisingly, the other three AGC3 kinases, and also D6PK reduced vacuolar targeting independent of the phosphorylation of the TPRXS motif serines (Fig. 7A). As we know that for PID, WAG1 and WAG2 the phosphorylation specificity of the PIN1HL is identical, it is likely that WAG1 and WAG2 act independent of PIN phosphorylation. For D6PK we cannot exclude that other phosphorylation events in the PIN1HL inhibit PIN1 vacuolar targeting. Strikingly, co-expression of the D6PKL kinases did not result in a significant reduction of PIN vacuolar targeting (a very mild effect was observed with D6PKL3). All kinases tested did however enhance accumulation of PIN1-GFP in endosomal structures independent of TPRXS phosphorylation, something we also observed for PID in Fig. 6A. We were able to show that a Phototropin kinase domain could not cause such an increase in endosomal structures (Fig. S3).

Discussion

Previously, we have shown that phosphorylation of PINs at the central serine residue in three conserved TPRSX(N/S) motifs by AGC3 kinases directs the trafficking of PIN proteins to the GNOM-independent pathway (Dhonukshe et al., 2010; Ding et al., 2011; Friml et al., 2004; Huang et al., 2010; Kleine-Vehn et al., 2009). Here we show that the AGC3 kinases, in addition to their role in PIN polarity regulation, also determine the PM abundance of PINs, in line with their previously proposed role as positive regulators of PAT (Benjamins et al., 2001; Lee and Cho, 2006). Vacuolar targeting and degradation of PIN1 may allow the plant to control auxin flux towards the root. Full phosphorylation at the three TPRS sites is necessary for shootward targeting of PIN1 and PIN2 (Dhonukshe et al., 2010; Huang et al., 2010) and our results show that this also prevents vacuolar targeting of PIN1 and PIN2. This positive effect of phosphorylation by AGC3 kinases is dose dependent, with increasing vacuolar signal and polarity loss of PIN1-GFP when single, double and triple serine to alanine mutants are observed. Interestingly, PID is not able to inhibit vacuolar targeting of PIN1-GFP S1,2,3A, while WAG1, WAG2, AGC3-4 and D6PK are (Fig. 7A). We have shown that PID can still phosphorylate PIN1-GFP S1,2,3A and enhance its presence in membrane compartments (Fig. 6B-D), however this extra phosphorylation is not able to influence PIN1-GFP S1,2,3A vacuolar targeting. Both the D6PK's and the AGC3 kinases enhance the endosomal accumulation of PIN1-GFP in protoplasts, while D6PKL1, 2 and 3 do not affect vacuolar targeting. This raises the possibility that there are certain phosphorylation sites

that might enhance trafficking of PINs to early endosomes, late endosomes and MVB's and do not enhance vacuolar targeting, instead leading to the observed buildup of PIN1-GFP due to excess kinase activity. Recently described S4 and S5 phosphorylation sites targeted by D6PK (Zourelidou et al., 2014) might be used by the AGC3 and D6PK's to enhance PIN cycling. However, since it has been shown that PID can phosphorylate these sites (Zourelidou et al., 2014), it is unlikely that these sites account for the difference between PID and the other AGC3 and D6 protein kinases. Thus phosphorylation at other sites than S1-5 might be involved in this process. Another possibility may be that the kinases that effect PIN1-GFP S1,2,3A in vacuolar targeting do so in an indirect manner. AC3-4, WAG1 and WAG2 can all be found in the nucleus in protoplasts (Chapter 2) (Galván-Ampudia and Offringa, 2007). A PID ortholog in maize (BIF2) phosphorylates BARREN STALK 1, a bHLH-type transcription factor, to promote inflorescence development (Skirpan et al., 2008). In *Arabidopsis* nuclearly localized AGC1 and AGC3 kinases could in theory phosphorylate nuclear factors that promote PIN1 PM abundance.

Fig. 6 shows that not all PIN1-GFP proteins in the plasma membrane are phosphorylated, yet their abundance is increased due to PID co-transfection. This means that phosphorylation enhances the PM localization of PINs, but that phosphorylation is not required to keep PINs in that location, since a large part of PIN1-GFP in membrane fractions is apparently unphosphorylated and PIN1-GFP S1,2,3A can also still be found at the PM (although in lower amounts). PINs at the PM are decidedly less mobile than other proteins in the PM (Kleine-Vehn et al., 2011), so there might be stabilizing factors that immobilize PINs at the PM after phosphorylation by PID and subsequent shootward targeting. The question of how the plant cell distinguishes between partial and fully phosphorylated PIN HLs is tied in with this. One possibility may be that conformational changes of the hydrophilic loop due to phosphorylation might change the way the hydrophilic loop interacts with possible receptors that control the cycling of PINs. A possible candidate for this proposed receptor function might be the MAB4/MEL family of proteins, since mutants of this family have shown increased PIN2 endocytosis and decreased PIN1 and PIN2 PM abundance and polarity (Furutani et al., 2011; Furutani et al., 2014).

The hypothesis that we propose is as follows: Full phosphorylation by AGC3 kinases directs PINs to the PM, where they are stabilized by interactions with other proteins or by self-oligomerization. Removal of phosphorylation by phosphatases increases the chance that PINs are endocytosed. By targeting PINs to the PM and by their stabilization at the PM, the amount of PINs that can be targeted to the vacuole is strongly reduced and this would cause the inhibition of vacuolar targeting observed in our experiments. The stabilization of PINs at polar parts of the PM is a mechanism proposed to be important for their super-polar distribution (Kleine-Vehn et al., 2011), linking polarity and abundance control to a single mechanism, namely the polarity control of PIN proteins due to phosphorylation (Fig. 8).

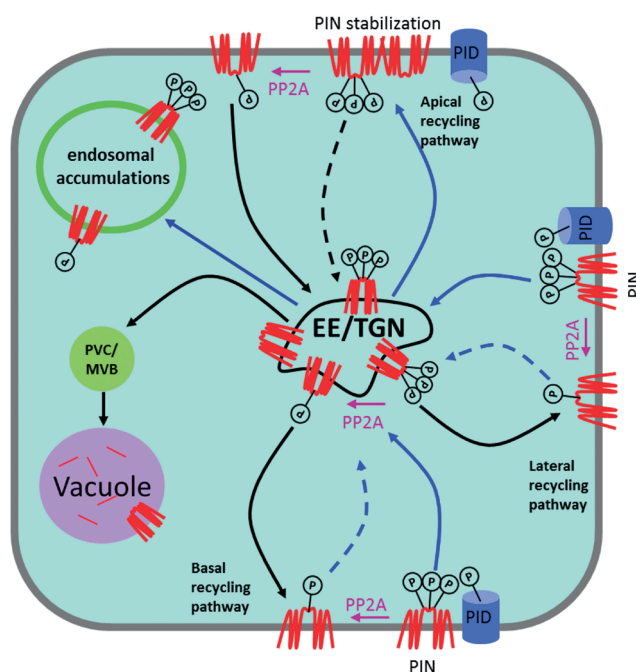


Figure 8: Model describing the dual role of PID as polarity determinant and enhancer of auxin efflux.

De novo synthesized PIN proteins arrive at the PM in a non-polar fashion. PIN phosphorylation at three TPRXS(N/S) motifs by the PM-associated PID kinase, directs PIN targeting from the basal to the apical recycling pathway, via the trans-golgi/recycling endosomes (TGN), finally reaching the apical PM. PINs that are only partially phosphorylated, due to the action of the PP2A phosphatases, are recruited back to early endosomes (EE) towards the basal or lateral recycling pathway. Both unphosphorylated and phosphorylated PINs can be found in PM fractions and PINs can remain there due to stabilization from self-oligomerization or through complex formation with other interactors. PINs can be targeted towards endosomal accumulations due to excess kinase activity and here PINs are not targeted towards the vacuole. Non-phosphorylated, non-stabilized PINs are targeted to the vacuole for degradation via the PVC/MVB.

In the *Arabidopsis* root meristems PIN1 and PIN2 have different localizations and distributions: PIN1 is largely basal and lateral and can be found in the developing vasculature, while PIN2 is apical in the epidermis and basal in the cortex. The apically localized PIN1 and PIN2 are probably fully phosphorylated at TPRXS(N/S) sites, while the basal PIN1 and PIN2 are probably not. However, it would be illogical if PIN1 and cortical PIN2 are fully dephosphorylated, since this would mean that they would be subject to constant vacuolar targeting and subsequent degradation. Somehow the non-PID AGC3 kinases and D6 protein kinases contribute to a mechanism that protects PINs from vacuolar targeting that is not linked to polarity control. It will be interesting to uncover what mechanism leads to this stabilization of PIN1 and PIN2.

For future work it would be interesting to investigate how phosphorylation of PINs is translated into different cycling pathways. Which complexes are recruited to cycle PINs to the rootward, lateral or shootward side and how do PIN PM micro-domains, detectable with super resolution microscopy (Kleine-Vehn et al., 2011), play a role in keeping PINs on the PM. Are they shielded from endocytosis by forming large oligomers or are interactors shielding them? Answering these questions will possibly solve the current missing link between PIN phosphorylation and the plasma membrane polarity or abundance output.

Materials and methods

Protoplast isolation and transformation

Arabidopsis thaliana Col-0 cell suspension cultures were used for protoplast preparations. Culture maintenance, protoplast isolation and transfections were performed as previously described (Schirawski et al., 2000) with minor modifications. Four-to-six days old cultures were diluted 5-fold in Cell Medium (30 g /L sucrose, 3.2 g/L Gamborg's B5 basal medium with mineral organics, adjusted to pH 5.8 with KOH and sterilized by autoclaving), incubated overnight and used for protoplast isolation. Following transfection with 10µg plasmid DNA per 1×10^6 protoplasts, the cells were incubated at 25 °C in the dark for 16-18 hours before observation or additional treatments. For quantification of PIN1-GFP vacuolar targeting dark-incubated protoplasts were observed using the regular mercury-lamp setup of the confocal microscope. Per treatment at least 40 protoplasts clearly expressing PIN1-GFP were scored for occurrence of vacuolar GFP signals. For the experiments in Fig. 6 and S2 protoplasts were isolated and transfected as described above. 20 hours after transfection protoplasts were spun down and the pellets flash frozen with liquid nitrogen followed by membrane/soluble fractions extraction as previously described (Abas and Luschignig, 2010).

DNA constructs and mutagenesis

The pBluescript-based *35S::PIN1-GFP* vector used for protoplast transformation was described before (Dhonukshe et al., 2007), and *pART7-35S::PID-mRFP* was constructed using the Gateway Technology (Invitrogen). The PID coding region was cloned into pDONOR207 by BP recombination, and subsequently introduced via LR recombination into a Gateway compatible pART7 destination plasmid containing the *CaMV35S* promoter and the gateway recombinant cassette in frame with the coding region of the *Monomer Red Fluorescent Protein (mRFP)* reporter gene. The *35S::PIN1-GFP* and *35S::PIN1-GFP S1,2,3A* constructs were described previously (Huang et al., 2010).

Plant materials and growth conditions

For all experiments, *Arabidopsis thaliana* ecotype Columbia 0 (Col-0) was used. *Arabidopsis* lines *PIN1-GFP*, *PIN1-GFP S1A*, *PIN1-GFP S1,3A*, *PIN1-GFP S1,2,3A*, *35S::PIN1-GFP*, *35S::PIN1-GFP S1,2,3A*, *PIN1-GFP S1,2,3E*, *eir1-1 PIN2::PIN2-YFP* and *eir1-1 PIN2::PIN2-YFP S1,2,3A* have been described previously (Dhonukshe et al., 2010; Huang et al., 2010). Seedlings were grown on MA medium (Masson and Paszkowski, 1992) at 21°C and a 16 hours photoperiod. Plants were grown on a mixture of 9:1 substrate soil and sand at 21°C, a 16 h photoperiod and 70% relative humidity. Transgenic tobacco BY-2 cell lines were obtained by *Agrobacterium*-mediated transformation and maintained as previously described (Adriana et al., 2003), using the previously described constructs *pINTAM>>PID* (Friml et al., 2004) and *PIN1::PIN1-GFP* (Benková et al., 2003). The *pINTAM>>PID PIN1::PIN1-GFP* doubly transformed cell line was used to measure the retention of ³H-NAA, as previously described (Zazimalová et al., 2007), following 48 hours pretreatment with or without 5 mM tamoxifen.

Confocal microscopy

Fluorescent signals in *Arabidopsis* roots and protoplasts were observed using a 20x or 40x objective of a ZEISS Axioplan LSM5 microscope equipped with a confocal laser scanning unit. GFP fluorescence was monitored with a 488 nm excitation and a 505-530 nm band pass emission filter. For the protoplast experiments. YFP was excited with a 514 nm argon laser and detected by a 530-560 nm band pass filter. mRFP was excited with a 543 nm argon laser and detected by 560 nm long pass filter. Transmitted light images were taken as a reference. PIN2-YFP signal intensity was measured using the open bioimage analysis platform ICY (icybioimageanalysis.org).

Proteins purification and western blot analysis

The protein purification and western blot analysis for Figures 6, S1 and S2 was performed according to a published protocol (Abas and Luschnig, 2010). For quantification of the bands in Fig. 6C The bioimage analysis program Fiji was used (Fiji.sc) and for the quantification performed in Fig. 6D we used the Biorad chemidoc XRS with Imagelab software.

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Supplemental data

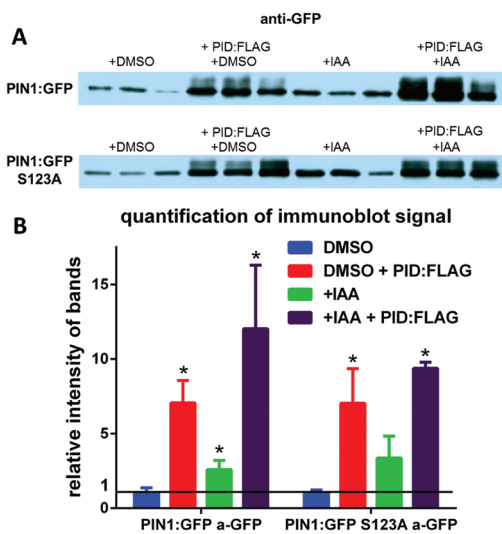


Figure S1: PIN1-GFP in *Arabidopsis* protoplasts is detected by an anti-GFP antibody in a similar manner as an anti-PIN1 antibody (See Fig. 6A and B).

(A) Western blot of membrane fractions of *Arabidopsis* protoplasts transfected with PIN1-GFP or PIN1-GFP S1,2,3A with or without IAA (1 μ M) and/or PID-FLAG and performed in triplo. Samples were probed with anti-GFP antibodies and repeat samples were loaded adjacent to each other. (B) Quantification of western blot in (A).

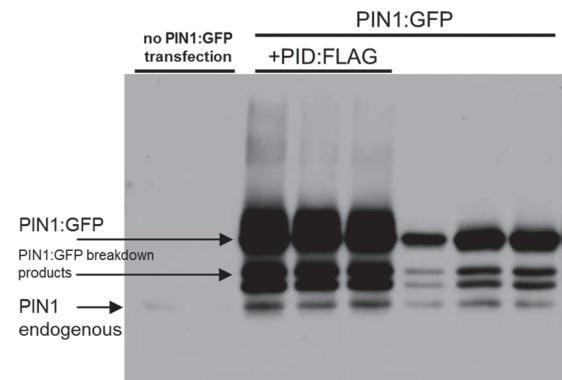


Figure S2: Endogenous PIN1 levels are significantly lower than PIN1-GFP levels. Western blot of membrane fractions from *Arabidopsis* protoplasts transfected with 35S::PIN1-GFP and 35S::PID:FLAG, probed with anti-PIN1. Without PIN1-GFP transfection a very vague band can be seen that corresponds to the expected MW of PIN1.

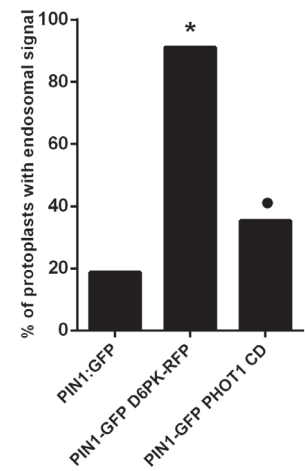


Figure S3: PIN1-GFP endosomal accumulation is specific for the AGC3 and D6PKs. *Arabidopsis* protoplasts transfected with PIN1-GFP and co transfected with D6PK display a strong increase in the number of cells with strong endosomal accumulations (As shown in Fig. 7D.). Phot1 catalytic domain (CD) co-transfection slightly increases the number of cells with these accumulations (Asterisk represents significance of $p < 0,001$ in a chi-square test compared to PIN1-GFP control and the closed circle represents a significance of $p = < 0,05 > 0,01$ compared to PIN1-GFP.)