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Components and targets of the PINOID signaling complex in *Arabidopsis thaliana*

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Citation

Zago, M. K. (2006, June 15). *Components and targets of the PINOID signaling complex in Arabidopsis thaliana*. Retrieved from <https://hdl.handle.net/1887/4436>

Version: Corrected Publisher's Version

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Note: To cite this publication please use the final published version (if applicable).

Chapter 4

**PINOID phosphorylates the PIN cytoplasmic loop at
multiple conserved serine residues**

Marcelo Kemel Zago and Remko Offringa

SUMMARY

The Arabidopsis PINOID (PID) protein serine/threonine kinase is a key regulator of auxin-mediated plant development, as threshold PID levels direct polar transport of auxin by determining the apico-basal polar targeting of the PIN auxin efflux transporters to the plasma membrane. The subcellular localization of animal transporters is known to be regulated by direct phosphorylation, mostly in a large cytoplasmic domain of these membrane proteins. Here we investigated the possibility that PIN proteins are direct phosphorylation targets of PID. *In silico* analysis of PIN1 revealed twenty-three putative phosphorylation sites, twenty-one of which are localized at the large cytoplasmic loop (CL) of this protein, and five of which are 100% conserved among the CL-containing PINs in Arabidopsis. *In vitro* assays using PID and synthetic PIN1 peptides containing most of the predicted phosphorylation sites identified four highly phosphorylated peptides comprising three of the predicted phosphorylated residues that are 100% conserved in the CL containing PINs. Notably, two of the strongly phosphorylated peptides comprise the T-P-R-X-S-N motif. By testing CLs of different PIN proteins and through site directed mutagenesis we deduced that the serines 231 and 290, both positioned in the conserved T-P-R-X-S-N motifs, are the major substrates for PID-mediated phosphorylation, and that the serines 377 and 380, that were previously shown to be phospho-substrates in PIN7 *in vivo*, may also be modified by PID. Our results suggest that the PID kinase affects PIN polarity through direct modification of multiple conserved serine residues in the large cytoplasmic loop of these auxin efflux facilitators.

Abbreviations: ARF-GEF, ADP-ribosylation factor-GTP exchange factor; BFA, Brefeldin A; F-actin, actin filament; GFP, green-fluorescent protein; GST, glutathione-S-transferase; IAA, indole-3-acetic acid; PAT, polar auxin transport; PBP, pinoid binding protein; PID, pinoid; PIN-CL, PIN cytoplasmic loop; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PM, plasma membrane

INTRODUCTION

The plant hormone auxin is an important regulator of plant development, and its - polar transport-driven - differential distribution in young growing organs is instrumental for a wide variety of developmental processes such as embryogenesis (1), root development (2), shoot organogenesis (3), and tropisms (4-6). The chemiosmotic model proposed for polar auxin transport (PAT) in the 1970s (7, 8), predicted that efflux carriers with a polar subcellular localization are essential for the direction of PAT, and thus for the positioning and maintenance of instructive auxin gradients in plants.

Molecular genetic studies in *Arabidopsis thaliana* identified the PIN family of membrane proteins as likely candidates for the auxin efflux carriers. These proteins were named after the *pin-formed* or *pin1* mutant, a loss-of-function mutant in the *PIN1* gene that is defective in PAT and develops pin-shaped inflorescences. The *Arabidopsis* genome encodes seven PIN1 homologs that have been named PIN2 to PIN8. PIN proteins contain two sets of five transmembrane domains that are linked by short and moderately conserved hydrophilic loops. In six of the PIN proteins the two transmembrane regions flank a large central hydrophilic cytoplasmic loop which contains several conserved stretches (4, 9). This domain structure is typical for proteins involved in transmembrane transport processes, and to date there is reasonably convincing evidence for the actual transport function of PIN proteins (10, 11). It has also been shown that PIN polar subcellular localization at the plasma membrane (PM) correlates well with the direction of PAT, and that their proper positioning is crucial for the correct directionality of the transport of auxin (1, 12, 13). Studies on the expression and subcellular localization of the different PIN proteins have drawn a complex picture that highlights specific roles for most of the PINs in auxin circulation and redistribution. The borders of action of each PIN, however, are far from being defined, and their functions commonly overlap. For example, it has been demonstrated that some PINs have their expression either enhanced and/or broadened to different cell files in the root tip in other *pin* loss-of-function backgrounds (14). This explains in part the observed functional redundancy among the different *PIN* genes (15-17).

The polar localization of PIN1 appears to primarily depend on the actin cytoskeleton (F-actin). It has been shown that the asymmetric localization of PIN1 in the PM is reduced in response to treatment with actin depolymerizing drugs. Interestingly, this treatment impairs PAT, corroborating the importance of F-actin and polar localization of PIN1 for this process. Actin depolymerization also prevents the internalization of PIN1 to endosomal compartments upon treatment with the vesicle trafficking inhibitor Brefeldin A (BFA), and the restoration of PIN1 localization after BFA wash-out, indicating that F-actin provides tracks for vesicle movement between the endosomal compartments and the PM (39). The ADP-Ribosylation Factor-GTP Exchange Factor (ARF-GEF) membrane protein GNOM was shown to be the BFA sensitive component that is required for recycling of PIN1 to the PM (18, 19). It remains to be established, however, whether GNOM is the polarity determinant in the recycling of PIN vesicles.

A true regulator of PIN polar targeting was identified through the *Arabidopsis pinoid* loss-of-function mutant that phenocopies the *pin formed* mutant. The *PINOID* gene was found to encode a protein serine/threonine kinase (20, 21) that determines the direction of PAT by regulating the subcellular polar localization of PIN proteins (22). Overexpression of *PID* results in targeting of PINs to the upper (apical) side of cells

in the root meristem, whereas PIN1 accumulates at the lower (basal) side of cells in the shoot meristem of *pid* loss-of-function mutants.

The fact that ectopic PID expression induces apical targeting of several PIN proteins suggests that the PID-dependent pathway recognizes a common feature in the PIN proteins. One possibility is that PID regulates an intermediate factor that in turn alters the polar targeting of PINs. The most attractive hypothesis, however, is that PID regulates the polar localization of PINs, and thereby the direction of PAT, through phosphorylation of PIN proteins. An interesting analogy exists between PID-dependent PIN polar localization in plant cells and signaling involved in polar deployment of transporters in animal cells. For example, asymmetric dispatch of the glucose transporter GLUT4 through secretory vesicles (GLUT4 secretory vesicles, or GSVs) has been demonstrated to be dependent on Protein Kinase C (PKC) phosphorylation of one particular GSV component, the insulin-responsive aminopeptidase (IRAP), at its amino terminal cytoplasmic loop (23). PKC has also been observed to mediate biphasic phosphorylation of the serotonin transporter (SERT), probably leading to SERT's silencing and subsequent internalization (24). It has also been shown that internalization of the dopamine transporter (DAT) is accelerated upon PKC activation, ultimately resulting in DAT accumulation in recycling endosomes (25). Finally, cAMP-dependent protein kinase (PKA) phosphorylation of the carboxy-terminal cytoplasmic loop of Aquaporin 2 (AQP2) has been demonstrated to be essential for the vesicle transport-mediated exocytosis of this water transporter to the apical membrane of renal duct cells (26, 27). Considering that PID and family members represent the likely plant orthologs of animal PKAs and PKCs (Galvan-Ampudia & Offringa, unpublished data) (28), it is possible that PID targets polar localization of PINs through phosphorylation of the central cytoplasmic loop of these proteins.

In this chapter we show that PID phosphorylates PIN proteins in the large cytoplasmic loop *in vitro*. By using deletion versions and generating single amino acid substitutions in the PIN1 loop, we were able to demonstrate that multiple conserved serine residues are targets for PID phosphorylation *in vitro*.

MATERIALS AND METHODS

Molecular cloning and constructs

Molecular cloning was performed following standard procedures (29). The generation of the yeast two-hybrid constructs pAS2-PID and pACT2-PBP2, the GST-PID construct (30, 31) and the histidine tagged-PIN fusions pET-PIN1CLsv (9), pET-PIN3CL (5), pET-PIN4CL (32) and pET-PIN7CL (1) and pGEX-PIN2CL (33) have been described previously. The yeast two-hybrid plasmid pACT2-PIN1CL and the pET-PIN6CL construct were kindly provided by Dr. Klaus Palme (Freiburg University, Germany) and Dr. Jiri Friml (University of Tübingen, Germany), respectively. The histidine tagged PID construct was created by digesting the *PID* cDNA with *XmnI*-*Sall* enzymes from a pBluescriptSK+-PID plasmid (31) and

cloned into pET16H (pET16B derivative, J. Memelink, unpublished results) treated with *Bam*HI and blunted and subsequently treated with *Xho*I. The GST-PIN1CL fusion was generated by cloning the *PIN1CL Smal/Sall* fragment from pACT2-PIN1CL into the corresponding restriction sites in plasmid pGEX-KG (34).

Yeast two-hybrid interaction

Using the Matchmaker II yeast two-hybrid system and *Saccharomyces cerevisiae* strain PJ69-4A (Clontech), PBP2 and PIN1CL fused to the GAL4 activation domain (pACT2) were directly tested at 20°C for interaction with PID fused to the GAL4 DNA binding domain (pAS2).

In vitro pull down experiments

GST tagged full-length PID or GST protein alone were used in pull down assays with histidine (his)-tagged PIN1CLsv (H-PIN1CLsv). Cultures of *E. coli* strain BL21 containing one of the constructs were grown at 37°C to OD₆₀₀ 0,8 in 50 ml LC supplemented with antibiotics. The cultures were then induced for 4 hours with 1 mM IPTG at 30°C, after which cells were harvested by centrifugation (10 min. at 4.000 RPM in tabletop centrifuge) and frozen overnight at -20°C. Precipitated cells were re-suspended in 2 ml Extraction Buffer (EB: 1x PBS, 2 mM EDTA, 2 mM DTT, supplemented with 0,1 mM of the protease inhibitors PMSF - Phenylmethanesulfonyl Fluoride, Leupeptin and Aprotinin, all obtained from Sigma) for the GST-tagged proteins or in 2 ml Binding Buffer (BB: 50 mM Tris-HCl pH 6,8, 100 mM NaCl, 10 mM CaCl₂, supplemented with PMSF 0,1 mM, Leupeptin 0,1 mM and Aprotinin 0,1 mM) for the his-tagged PIN1CLsv and sonicated for 2 min. on ice. From this point on, all steps were performed at 4°C. Eppendorf tubes containing the sonicated cells were centrifugated at full speed (14.000 RPM) for 20 min., and the supernatants were transferred to fresh 2 ml tubes. H-PIN1CLsv supernatant was left on ice, while 100 µl pre-equilibrated Glutathione Sepharose resin (pre-equilibration performed with three washes of 10 resin volumes of 1x PBS followed by three washes of 10 resin volumes of 1x BB at 500 RCF for 5 min.) was added to the GST- fusion protein containing supernatants. Resin-containing mixtures were incubated with gentle agitation for 1 hour, subsequently centrifugated at 500 RCF for 3 min. and the precipitated resin was washed 3 times with 20 resin volumes of EB. Next, H-PIN1CLsv supernatant (approximately 2 ml) was added to GST-fusion-containing resins, and the mixtures were incubated with gentle agitation for 1 hour. After incubation, supernatants containing GST resins were centrifugated at 500 RCF for 3 min., the new supernatants were discarded and the resins subsequently washed 3 times with 20 resin volumes of EB. Protein loading buffer was added to the resin samples, followed by denaturation by 5 min. incubation at 95°C. Proteins were subsequently separated on a 12% polyacrylamide gel prior to transfer to an Immobilon™-P PVDF (Sigma) membrane. Western blots were hybridized using a horse radish peroxidase (HRP)-conjugated anti-pentahistidine antibody (Quiagen) and detection followed the protocol described for the Phototope-HRP Western Blot Detection Kit (New England Biolabs).

In vitro phosphorylation assays

Proteins used in *in vitro* phosphorylation assays were his- or GST- tagged for purification from several (usually five) aliquots of 50 ml cultures of *E. coli*. strain BL21 which were grown, induced, pelleted and frozen as described above for the *in vitro* pull down experiments. Each aliquot of frozen cells pellet was resuspended in 2 ml Lysis Buffer (25 mM Tris-HCl pH 8,0; 500 mM NaCl; 20 mM Imidazol; 0,1% Tween-20; supplemented with 0,1 mM of the protease inhibitors PMSF, Leupeptin and Aprotinin) for the his-tagged proteins, or in 2ml of EB (supplemented with 0,1 mM of the protease inhibitors PMSF, Leupeptin and Aprotinin) for the GST-tagged proteins, and subsequently sonicated for 2 min. on ice. From this point on, all steps were performed at 4°C. Sonicated cells were centrifugated at full speed (14.000 RPM) for 20 min, the new pellets were discarded, and supernatants from all aliquots of the same construct were transferred to a 15 ml tube containing 100 µl of pre-equilibrated Ni-NTA resin (pre-equilibration performed with three washes of 10 resin volumes of Lysis Buffer at 500 RCF for 5 min.) for the his-tagged proteins or 100 µl of pre-equilibrated (see above) Glutathione Sepharose for the GST-tagged proteins.

Supernatants and resins were incubated with gentle agitation for 1 hour. After incubation, supernatants containing resins were centrifuged at 500 RCF for 3 min., the new supernatants were discarded and the resins subsequently washed: 3 times with 20 resin volumes of Lysis Buffer, once with 20 resin volumes of Wash Buffer 1 (25 mM Tris.Cl pH 8,0; 500 mM NaCl; 40 mM Imidazol; 0,05% Tween-20) and once with 20 resin volumes of Wash Buffer 2 (25 mM Tris-HCl pH 8,0; 600 mM NaCl; 80 mM Imidazol) for the his-tagged proteins; 3 times with 20 resin volumes of EB for the GST-tagged proteins. In between the washes, the resins were centrifuged for 5 min. at 500 RCF. After the washing steps, 20 resin volumes of Elution Buffer (25 mM Tris.HCl pH 8,0; 500 mM NaCl; 500 mM Imidazol) was added to the Ni-NTA resin and incubated for 15 min; the resin was subsequently centrifuged for 3 min. at 500 RCF and the supernatant containing the desired protein transferred to a new tube. For the GST-tagged proteins, the elution was performed by adding to the Glutathione Sepharose resin 3 resin volumes of Glutathione Elution Buffer (Reduced Glutathione 10 mM, Tris-HCl pH 8,0 50 mM), the mixture was gently agitated for 10 min at R.T., the resin was subsequently centrifuged for 3 min. at 500 RCF and the supernatant containing the desired protein transferred to a new tube; this process was repeated twice more. The solutions containing the proteins were diluted a 1000-fold in Tris Buffer (25 mM Tris.HCl pH7,5; 1 mM DTT) and concentrated to a workable volume (usually 50 µl) using Vivaspin microconcentrators (10 kDa cut off, maximum capacity 600 µl, manufacturer: Vivascience). Glycerol was added as preservative to a final concentration of 10% and samples were stored at -80°C.

Approximately 1 µg of each purified protein (PID and substrates) in maximal volumes of 10 µl were added to 20 µl kinase reaction mix, containing 1x kinase buffer (25 mM Tris-HCl pH 7,5; 1 mM DTT; 5 mM MgCl₂) and 1 x ATP solution (100 µM MgCl₂/ATP; 1 µCi ³²P-γ-ATP). Reactions were incubated at 30°C for 30 min. and stopped by the addition of 5 µl of 5 x protein loading buffer (310 mM Tris-HCl pH 6,8; 10 % SDS; 50% Glycerol; 750 mM β-Mercaptoethanol; 0,125% Bromophenol Blue) and 5 min. boiling. Reactions were subsequently separated over 12,5% acrylamide gels, which were washed 3 times for 30 min. with kinase gel wash buffer (5% TCA – Trichloroacetic Acid; 1% Na₂H₂P₂O₇), coomassie stained, destained, dried and exposed to X-ray films for 24 to 48 hours at -80°C using intensifier screens. For the peptides assays, 1µg of purified PID was incubated with 4 nmol of 9^{mer} biotinylated peptides (Pepscan) in a phosphorylation reaction as described above. Reaction processing, spotting and washing of the SAM² Biotin Capture Membrane (Promega) were performed as described in the corresponding protocol. Following washing, the membranes were wrapped in plastic film and exposed to X-ray films for 24 to 48 hours at -80°C using intensifier screens. The phosphorylation intensities of each peptide were determined by densitometry analysis of the autoradiographs using the ImageQuant software (Molecular Dynamics).

Site directed mutagenesis

For the site directed mutagenesis we used the Quickchange XL site directed mutagenesis kit (Stratagene). The oligonucleotides used to introduce mutations in the *PIN1CL* cDNA were 5'-CGACACCTAGACCTGCGAATCTAACCAACG-3' and 5'-CGTTGGTTAGATTGCGAGGTCTAGGTGT CG-3' to change the serine 231 for alanine, 5'-CCTACTCCGAGACCTGCCAACTACGAAGAAG-3' and 5'-CTTCTTCGTAGTTGGCAGGTCTCGGAGTAGG-3' to change serine 290 for alanine, 5'-GGCTT ATCTGCGGCACCTAGACC-3' and 5'-GGTCTAGGTGCCGAGATAAGCC-3' to change threonine 227 for alanine, 5'-GGCTTATCTGCGGCACCTAGACCTGCGAATCTAACCAACG-3' and 5'-CGTTGGTT AGATTGCGAGGTCTAGGTGCCGAGATAAGCC-3' to replace both threonine 227 and serine 231 for alanines, 5'-GGTTCTAAAGGTCTGCTCCGAGACCTTCC-3' and 5'-GGAAGGTCTCGGAGCAGGACC TTTAGAACC-3' to replace threonine 286 for alanine, and 5'-GGTTCTAAAGGTCTGCTCC GAGACCTGCCAACTACGAAGAAG-3' and 5'-CTTCTTCGTAGTTGGCAGGTCTCGGAGCAGGACC TTTAGAACC-3' to replace threonine 286 and serine 290 for alanines at once.

The oligonucleotides used to introduce mutations in the *PIN1CLsv* cDNA were 5'-CGAATT CTAATCGAGACCTGCCAACTACGAAGAAGAC-3' and 5'-GTCTTCTTCGTAGTTGGCAGGTCTCGA GTAGAATTCG-3' to replace serine 290 for alanine.

RESULTS

The PIN1 cytoplasmic loop is a likely target for phosphorylation

The previous observations that PID activity affects PIN polar targeting (22), and that phosphorylation of transporters is a signal for endo- or exocytosis in animal cells (23, 26, 27, 35), led us to investigate whether PIN proteins are phosphorylation targets of the PID protein kinase. The fact that PINs contain transmembrane domains (Figure 1A), as predicted by Predictprotein (36), precludes the use of the complete proteins in *in vitro* phosphorylation assays. As an alternative approach we used the NetPhos program (37) to first identify putative phosphorylation sites in PIN1. This identified twenty-three possible phosphorylation sites, twenty-one of which are located in the large cytoplasmic loop of PIN1 (Figure 1B). Since trafficking-related phosphorylation of animal transporters is known to occur in the large cytoplasmic domain of these proteins (23, 26, 27, 35), we decided to focus our analysis on the cytoplasmic loop of PIN1 (PIN1CL).

PINOID does not show a strong interaction with the PIN1 cytoplasmic loop

First, we tested the physical interaction between PID and its putative phospho-target PIN1CL. Two yeast plasmids, the bait encoding PID fused to the GAL4 DNA binding domain (BD), and the prey encoding a fusion between PIN1CL and the GAL4 activation domain (AD), were co-introduced in the yeast strain PJ69-4A. A prey plasmid encoding the PID Binding Protein 2 (PBP2) GAL4 AD fusion was used as a positive control in this yeast two-hybrid experiment (Figure 1C). In a parallel approach, we tested *in vitro* pull down of an his-tagged shorter version of PIN1CL (PIN1CLsv) with GST-tagged PID (Figure 1D). Neither of the two approaches detected a direct interaction between PID and PIN1CL. The interaction between the PID protein serine/threonine kinase and its substrates may be very transient, and it could be that such weak interactions are not detected using the above-mentioned methods. Moreover, we can not exclude that PID interacts with other regions of PIN1 protein or that PID needs accessory proteins for its interaction with PIN1.

PID phosphorylates the cytoplasmic loop of PIN proteins *in vitro*

Next we co-incubated histidine-tagged PID with GST-tagged PIN1CL in an *in vitro* phosphorylation reaction. Following separation of the proteins on gel and autoradiography, clear PID-dependent phosphorylation of the PIN1CL was detected (Figure 1E). In order to map the PID substrates in PIN1CL, we synthesized seventeen PIN1CL-specific peptides containing all the twenty-one putative phosphorylation sites predicted by NetPhos (Figure 1B) (37). From these, only twelve peptides could be used in our assay, as the others could not be dissolved in the reaction buffer. The twelve soluble peptides represent seventeen of the putative

phosphorylation sites. *In vitro* phosphorylation reactions revealed that peptides 2, 6, 11 and 12, respectively corresponding to the sequences GLSATPRPS, TPRPSNYEE, VMPPTSVMT and RNPNSYSS, were most intensely phosphorylated by PID (Figure 1F).

Since the PID-dependent switch in basal-to-apical polar targeting is not restricted to PIN1, but has also been observed for PIN2 and PIN4 (22), we aligned the cytoplasmic loops of PIN1, 2, 3, 4, 6 and 7 to identify the conserved serine- and threonine residues. Indeed several of such residues appeared to be fully conserved in the different CLs, although only five of them were predicted by the NetPhos program (37) to be putative phosphorylation sites (Figure 2). Interestingly, three of the five conserved and predicted phosphorylation sites are located in two TPRXSN motifs represented by peptides 2 (GSATPRPS) and 6 (TPRPSNYEE) that were highly phosphorylated by PID (Figure 1F and 2).

To test whether PID also phosphorylates the CLs of other PIN proteins, and to identify the phosphorylated residues, we performed new phosphorylation assays using his- or GST-tagged versions representing different portions of the CLs loops of PIN1 (PIN1CLsv), PIN2 (PIN2CL), PIN3 (PIN3CL), PIN4 (PIN4CL), PIN6 (PIN6CL) and PIN7 (PIN7CL) (Figure 3A). Notably, those PINCLs containing one or both of the conserved TPRXSN motifs were phosphorylated, whereas the PINCLs lacking these motifs showed much reduced phosphorylation levels (Figures 3B and 3C). These results suggested that the two conserved TPRXSN motifs represent the most significant targets in the PINCLs for post-translational modification by the PID protein kinase.

PID phosphorylates multiple conserved residues in the PINCL

In the view of the previous observations, we created the GST-tagged PIN1CL mutants PIN1CL^{S231A S290A}, PIN1CL^{T227A S231A S290A} and PIN1CL^{T227A S231A T286A S290A}, in which the serines and threonines in the two TPRXSN motifs were replaced by alanines. The mutations S231A and S290A resulted in a significant reduction in phosphorylation of PIN1CL (Figure 3D), whereas the mutations T227A and T286A did not cause a further reduction of the phosphorylation signal (Figure 3E). This result supports the previous conclusion that the conserved TPRXSN motifs represent important PID phospho-substrates in the PINCLs. However, the phosphorylation levels observed in the mutant PIN1CLs are still considerable (Figures 3D and 3E), suggesting that additional serine or threonine residues are phosphorylated by PID.

Considering that the GST-PIN1CL and GST-PIN1CL^{S231A S290A} proteins produce several breakdown products that are differentially phosphorylated (Figures 1E and 3D), we used these characteristics to estimate the position of other putative PID

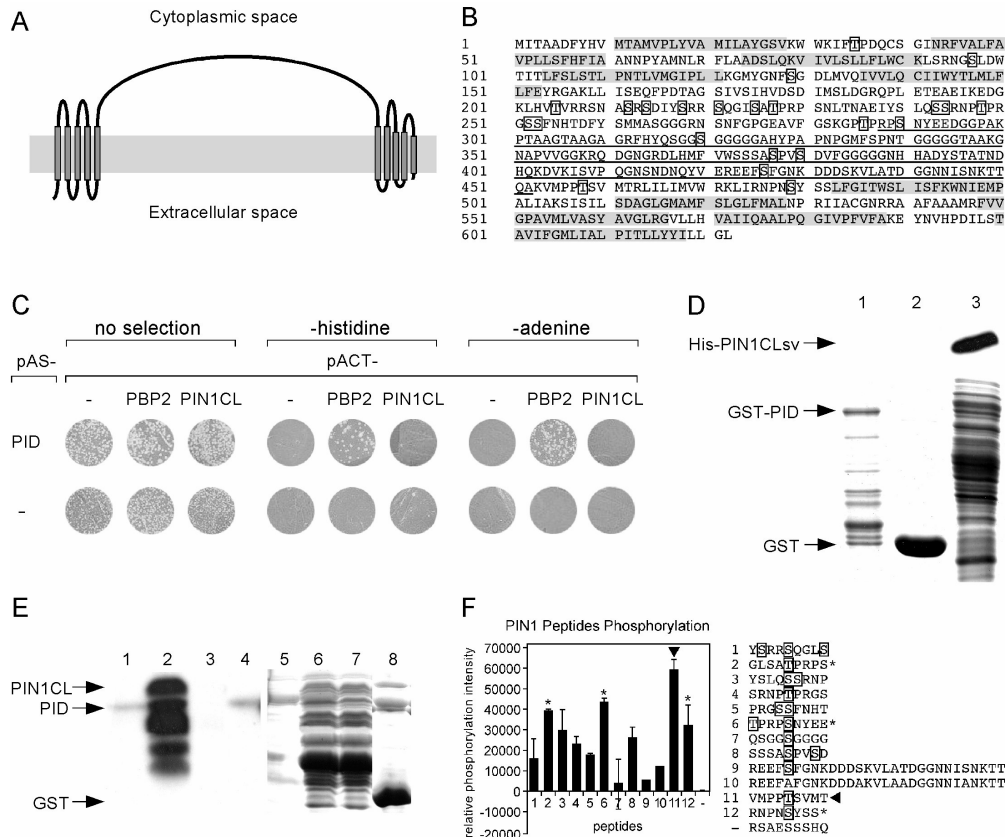


Figure 1. PID phosphorylates but does not tightly interact with the PIN1 cytoplasmic loop. (A) Schematic representation of the PIN1 protein, as predicted by the PredictProtein Software (Rost *et al.*, 2004). (B) Amino acid sequence of the PIN1 protein, with the transmembrane regions indicated by gray shading. The PIN1 cytoplasmic loop (PIN1CL) used for the yeast two-hybrid analysis and *in vitro* phosphorylation assays comprises the region within amino acid residues 159 and 482. The NetPhos (Blom *et al.*, 1999) predicted putative phosphorylation sites are boxed and the short version of the PIN1 cytoplasmic loop (PIN1CLsv) used in the *in vitro* protein pull-down and phosphorylation assays is underlined. (C) Yeast two-hybrid assay using PID, PID Binding Protein 2 (PBP2), PIN1CL and empty vectors as controls in non-selective medium and medium lacking histidine or adenine. (D) Immunodetection with anti-his antibodies (top panel) and coomassie stained gel (bottom panel) of an *in vitro* protein pull-down assay using histidine-tagged PIN1CLsv together with GST-tagged PID (lane 1) or GST- protein alone (lane 2). Total protein extract (1% of input) of H-PIN1CLsv (lane 3) was used as control. (E) Autoradiograph (lanes 1 to 4) and coomassie stained gel (lanes 5 to 8) showing that GST-PIN1CL (lanes 2, 3, 6 and 7) is phosphorylated in the presence of his-PID (lanes 1, 2, 4, 5, 6 and 8). Absence of phosphorylation in the GST protein alone (lanes 4 and 8) indicates specific PID-mediated modification of the PIN1CL portion in the fusion protein GST-PIN1CL. (F) *In vitro* assay showing the relative phosphorylation intensities of biotinylated PIN1 peptides containing putative phosphorylation sites (boxed). Highly phosphorylated PIN-conserved and PIN1-specific peptides are indicated by stars and the arrowhead, respectively. The peptide RSAESSHQ was used as negative control (-).

PID phosphorylates PIN cytoplasmic loops at conserved residues

PIN1CL	GAKLLISEQF	P.DTAGSIVS	IHVDSDIMSL	DGRQPLETEA	EIKEDGKLHV
PIN2CL	GAKLLISEQF	P.ETAGSITS	FRVDSDVISL	NGREPLQTD	EIGDDGKLHV
PIN3CL	GAKMLIMEQF	P.ETAASIVS	FKVESDVVSL	DGHDFLETD	EIGDDGKLHV
PIN4CL	GAKLLIMEQF	P.ETGASIVS	FKVESDVVSL	DGHDFLETD	EIGNDGKLHV
PIN6CL	AARLLIRAEF	PGQAAGSIK	IQVDDDVISL	DGMDPLRTET	ETDVNGRIRL
PIN7CL	GAKILIMEQF	P.ETGASIVS	FKVESDVVSL	DGHDFLETD	QIGDDGKLHV
PIN1CL	TVRRSNASRS	DIYSR.....	.RSQGLSATP	RPSNLTNAEI	YSLQSSRN.P
PIN2CL	VVRRSSAASS	MISSFNKSHG	GGLNSSMITP	RASNLTGVEI	YSVQSSRE.P
PIN3CL	TVRKSNASRR	SFCG.....PNMTP	RPSNLTGAEI	YSLST.....
PIN4CL	TVRKSNASRR	SLM.....MTP	RPSNLTGAEI	YSLSS.....
PIN6CL	RIRRSVSSVP	DSVMSS.....SLCLTP	RASNLSNAEI	FVSNTPNNR
PIN7CL	TVRKSNASRR	SFYGG.....	.G..GTNMT	RPSNLTGAEI	YSLNT.....
PIN1CL	TPRGSSFNHT	DFYSMMAS..GGGRNSNFG	PGEAVFG...
PIN2CL	TPRASSFNQT	DFYAMFNASK	APSPRHGYTN	SYGGAGAGPG	.GDVYSLOS.
PIN3CL	TPRGSNFNHS	DFYNNMGF..PGGRLSNFG	PADMYSVQS.
PIN4CL	TPRGSNFNHS	DFYSVMGF..PGGRLSNFG	PADLYSVQS.
PIN6CL	FHGGGGSGTL	QFYNGSNE..IM	FCNGDLGGFG	FTRPGLGASP
PIN7CL	TPRGSNFNHS	DFYSMMGF..PGGRLSNFG	PADMYSVQS.
PIN1CLSKGFTPRP	SNYEEDGGPA	KPTAAGTAAG	AGRFHYQSGG
PIN2CLSKGVTPT	SNFDEEVMT	AKKAGRGGR.SM
PIN3CLSRGFTPRP	SNFEENCAMA	SSPRFGYY..
PIN4CLSRGFTPRP	SNFEENNAV.	...KYGFYNN	T....NSSV
PIN6CL	RRLSGYASSD	AYSLOFTPR	SNFNELDVN.
PIN7CLSRGFTPRP	SNFEESCAMA	SSPRFGYY..
PIN1CL	SGGGGG....	AHYAPNPGM	FSPNTGGGG	TAAKGNAPVV	GGKROD....
PIN2CL	SGELYNNNSV	PSYPPNPMF	TGSTSGASGV	KKKESGGGGS	GGGVGVG...
PIN3CL	PGGGA....	GSYPAPNPEF	SSTTTSTA.N	.KSVNKNPKD	VNTNQQTLP
PIN4CL	PA..A....	GSYPAPNPEF	S...TGTGVS	TKP.NKIPKE	.N.QQQ..LQ
PIN6CL	GN....G..	TPVWMKSPAA	GRIYRQS...
PIN7CL	PGG.AP....	GSYPAPNPEF	S....TG.N	.KTGSKAPKE	.N.HHH..V.
PIN1CLGN...	.GRDLHMFVW	SSSASPVS	FGG....GGGNHADY
PIN2CL	..GQ..N...	..KEMNMFVW	SSSASPVSEA	NAKNAMTRGS
PIN3CL	TGGK..SN.SH	DAKELHMFVW	SSNGSPVSDR	A.GLNVFGGA	PDNDQGG..R
PIN4CL	..EKDSKASH	DAKELHMFVW	SSSASPVS	VFG....GGA	..GDNVATEQ
PIN6CLSPKMMW	E.....
PIN7CL	..GK..SN.SN	DAKELHMFVW	SSNGSPVSDR	A.GLQVDNGA	..NEQVG..K
PIN1CL	STATNDHQKD	VKISVPQGN.	SNDNQ.....
PIN2CL	S..T.DVSTD	PKVSIPPHDN	LATKAMQNLI	ENMSPGRKG.
PIN3CL	SDQG.AKEIR	MLVPDQSHNG	ETKAVAHPAS	G.DFGG....	EQQFSFAGKE
PIN4CL	SEQG.AKEIR	MVVSQDPRK.	SN.ARG...G	GDDIGG...L	D...S..G..
PIN6CL	SGQR.HAAKD	INGSVPEKEI	SFRDALKAAP	QATAAG....
PIN7CL	SDQGGAKEIR	MLISDHTQNG	ENKA.G.PMN	G.DYGG....	E.....
PIN1CL	.YVER..EEF	SFGNKDDDSK	VLATDGGN..	...NISNKT	QAK.VMPPTS
PIN2CL	.HVEMD...	QDGNNGGKSP	YMGKKGS	DG.GPG....	PRKQMPAS
PIN3CL	EEAER.PKDA	ENGLNKLAPN	STAALQSK..	TGLG.GAEAS	QRK.NMPAS
PIN4CL	.EGEREIEKA	TAGLNKMGSN	STAELEAAGG	DG.G.G.NNG	.T..HMPPTS
PIN6CLGGASM	EEGAAGKDTT	PVAAIG....	.KQ.EMPSAI
PIN7CL	EESER.VKEV	PNGLHKLRCN	STAE LNPK..	EAIETG.ETV	PVK.HMPAS
PIN1CL	VMTRLILIMV	WRKLIRNPNS	YSS		
PIN2CL	VMTRLILIMV	WRKLIRNPNT	YSS		
PIN3CL	VMTRLILIMV	WRKLIRNPNT	YSS		
PIN4CL	VMTRLILIMV	WRKLIRNPNT	YSS		
PIN6CL	VMMRLILT	VRKLIRNPNT	YSS		
PIN7CL	VMTRLILIMV	WRKLIRNPNT	YSS		

Figure 2. Conserved putative phosphorylation sites in the cytoplasmic loops of PINs. Residues labeled with black (serines and threonines) and light gray (other amino acids) shading are completely conserved in the Arabidopsis PINCLs; Serines or threonines that are conserved in four or five of the six PINCLs are indicated by dark gray shading. Conserved serines or threonines that are predicted to be phosphorylated by NetPhos (Blom *et al.*, 1999) are marked with a star. Boxes highlight conserved TPRXSN motifs. The PKC-like and MAPK phosphorylation sites detected by Nushe and co-workers (Nuhse *et al.*, 2004) are indicated by empty (except for the methionine) and filled arrowheads, respectively.

phosphorylation sites. First we predicted the sizes of the phosphorylated breakdown products based on the protein size marker (not shown) and the known sizes of the full-length GST-PIN1CL (60 kDa) and his-PID (53 kDa). For this analysis we assumed that, based on their purification with glutathione beads, each GST-PIN1CL breakdown product should at least comprise the GST moiety (27 kDa). The smallest phosphorylated GST-PIN1CL breakdown product was estimated to be 35 kDa with an 8 kDa N-terminal part of PIN1CL containing the first conserved TPRXSN motif with serine 231. This was confirmed by the observation that the corresponding part of the GST-PIN1CL^{S231A S290A} mutant protein is not phosphorylated (Figure 3D). The next phosphorylated GST-PIN1CL breakdown product was estimated to be 43 kDa, and should comprise a 16 kDa N-terminal PIN1CL portion containing the serines 231 and 290 of the two conserved TPRXSN motifs, peptide 3 (YSLQSSRNP) that was weakly phosphorylated by PID (Figure 1F), and a third less well conserved TPRXS motif containing serine 252 (Figures 1B and 2). The fact that the corresponding GST-PIN1CL^{S231A S290A} mutant breakdown product is only weakly phosphorylated (Figure 3D), suggests that serines 231 and 290 are the main phosphorylation substrates of PID, and that the serines in YSLQSSRNP or TPRGSS are only weakly contributing to PID-mediated phosphorylation. The next two strongly phosphorylated GST-PIN1CL sub-products are respectively 47 and 50 kDa and include two additional conserved serines 377 and 380 that are putative weak phosphorylation targets of PID, based on the phosphorylation signal obtained with the corresponding peptide 8 (SSSASPVSVD, Figure 1F). Interestingly, the same residues were shown to be *in vivo* phospho-substrates in PIN7 (38). Considering that the corresponding protein fragments derived from the mutant GST-PIN1CL^{S231A S290A} are efficiently phosphorylated (Figure 3D), it is likely that serines 377 and 380 are significantly modified by PID. Finally, the full length GST-PIN1CL and GST-PIN1CL^{S231A S290A} are efficiently modified by PID, but not more efficiently than the 47 and 50 kDa GST-PIN1CL break down products. The peptides 11 (VMPPTSVMT) and 12 (RNPNSYSS), which were observed to be very significantly phosphorylated by PID (Figure 1F), do not seem to significantly contribute to PID-mediated phosphorylation when in context of the full length cytoplasmic loop.

To narrow down the search for the other phospho-target sites of PID, further assays employed a shorter version of the PIN1CL (PIN1CLsv) and two of its mutant versions, PIN1CLsv^{S290A} in which serine 290 was replaced for an alanine, and PIN1CLsv^{ΔCT} lacking carboxy-terminal amino acids including serines-377 and -380 (Figure 3A). The results showed that PIN1CLsv and PIN1CLsv^{ΔCT} were still efficiently phosphorylated, but that PIN1CLsv^{S290A} was modified at a much reduced level (Figure 3F). In one hand, this confirmed that serine 290 is one of the most efficient targets of the PID kinase in the PIN1CLsv. On the other hand, the minor signal observed in PIN1CLsv^{S290A} indicates that other residues in this protein could still be phosphorylated at low levels. Based on the results obtained in the peptide experiments (Figure 1F), and on the analysis of the breakdown products of GST-PIN1CL, other putative phosphorylation targets of PID in PIN1CLsv^{S290A} could be the conserved serines 377 and 380. However, the significance of PID-mediated modification of these serines remains to be addressed.

DISCUSSION

Proper polar subcellular localization of PIN proteins in the plasma membrane of plant cells is essential for the correct directionality of PAT (12, 13, 17). Moreover, the apical-basal polar targeting of PINs has been demonstrated to be determined by the levels of activity of the serine/threonine kinase PID (22). PID belongs to the plant specific AGCVIII group of protein kinases (20, 21). Animal orthologs of these kinases are also known to play a role in regulating the membrane localization of transporter proteins by phosphorylation of their cytoplasmic loops (23, 26, 27). Therefore, it is conceivable that PID mediates its effect through phosphorylation of the cytoplasmic loop of PIN proteins.

PID-dependent phosphorylation of PINs may direct PIN apical targeting

Recently, several lines of evidence indicate that signals for the subcellular polar localization of PIN proteins are embedded in their amino acid sequence. For example, it has been shown that deployment of PIN1 to the correct cellular pole was affected by the position of the GFP insertion in the large cytoplasmic loop (13). Moreover, modification of a serine residue in one of the short cytoplasmic loops of PIN2 to a glycine resulted in failure of PIN2 deployment to the PM (11). Although these data do not clarify the particular signal responsible for PIN exocytosis, it is suggestive that protein modification, and possibly phosphorylation, events could be important for this process.

Accordingly, our own analysis shows that multiple putative phosphorylation sites are present in the large cytoplasmic loop that is present in six of the eight PINs, and that some of these sites are conserved among these proteins. Recently, Nuhse and co-

workers (38) showed that the cytoplasmic loop of the *Arabidopsis thaliana* PIN7 is phosphorylated *in vivo* in two conserved serines that were predicted as MAPK and PKC phosphorylation sites.

In the present work we show that PID is capable of phosphorylating different PINCLs *in vitro*. Our analysis suggests that PID modifies the serines 290 and 231

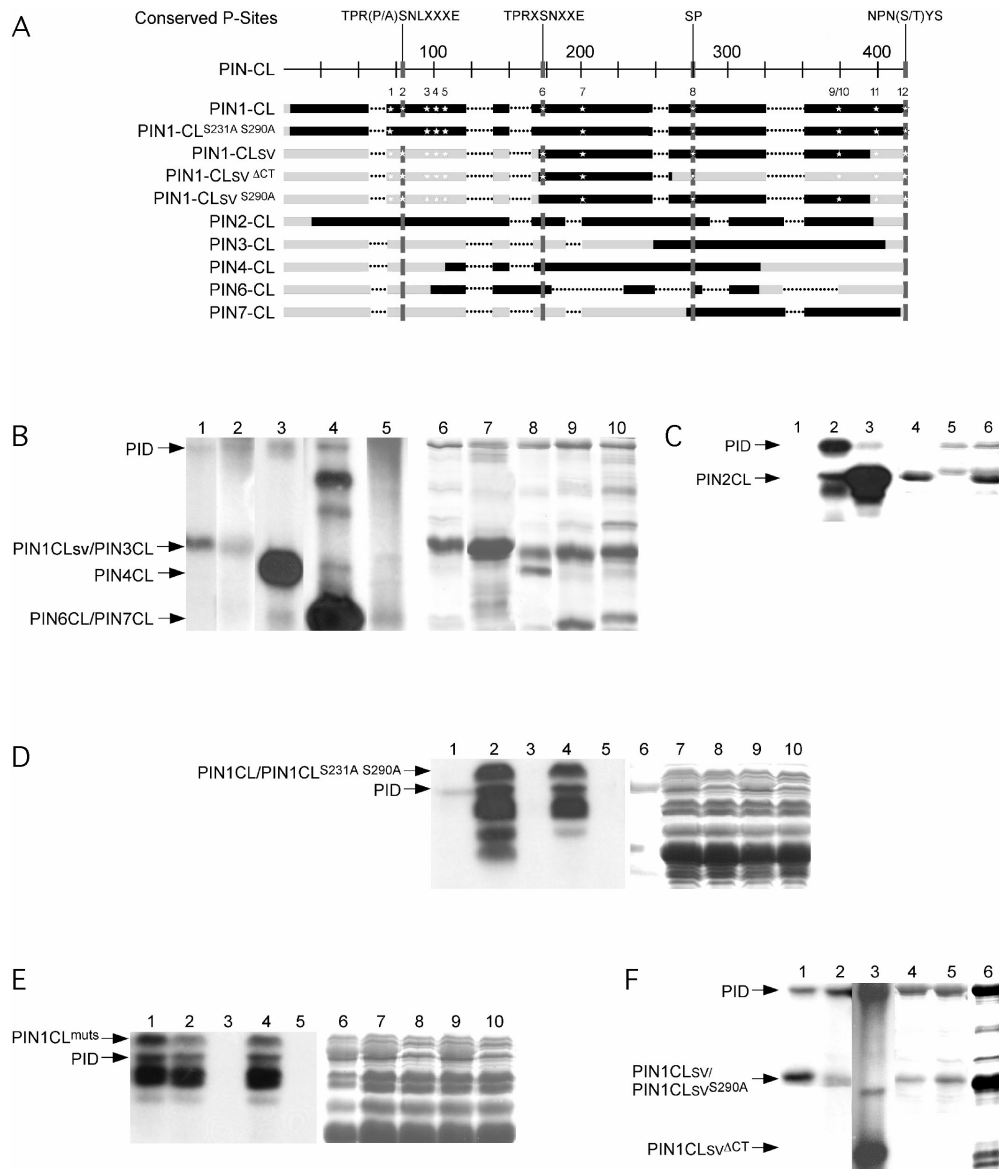


Figure 3. PID phosphorylates PINCLs in conserved sites. (A) Schematic alignment of the complete cytoplasmic loops of Arabidopsis PIN1, 2, 3, 4, 6 and 7. Thin punctuated lines indicate gaps in the alignment. The 100% conserved predicted phosphorylation sites are indicated with thin vertical dark gray bars. The parts of the CLs that were tested in the *in vitro* phosphorylation assays with PID are indicated with black bars. The positions of the peptides used in *in vitro* phosphorylation assays are indicated with stars in the PIN1CL. The corresponding numbers (see also Figure 1F) are provided above the alignment. (B) Autoradiograph (lanes 1 to 5) and coomassie stained gel (lanes 6 to 10) of an *in vitro* phosphorylation assay using his-PID (all lanes) together with his-PIN1CLsv (lanes 1 and 6), his-PIN3CL (lanes 2 and 7), his-PIN4CL (lanes 3 and 8), his-PIN6CL (lanes 4 and 9) or his-PIN7CL (lanes 5 and 10). (C) Autoradiograph (lanes 1 to 3) and coomassie stained gel (lanes 4 to 6) of an *in vitro* phosphorylation assay of GST-PID (lanes 2, 3, 5 and 6) together with GST-PIN2CL (lanes 1, 3, 4 and 6). (D) Autoradiograph (lanes 1 to 5) and coomassie stained gel (lanes 6 to 10) of an *in vitro* phosphorylation assay of his-PID (lanes 1, 2, 4, 6, 7 and 9) together with GST-PIN1CL (lanes 2, 3, 7 and 8) or GST-PIN1CL^{S231A S290A} (lanes 4, 5, 9 and 10). (E) Autoradiograph (lanes 1 to 5) and coomassie stained gel (lanes 6 to 10) of an *in vitro* phosphorylation assay of his-PID (lanes 1, 2, 4, 6, 7 and 9) together with the GST-PIN1CL mutants (PIN1CL^{mutS}) GST-PIN1CL^{S231A S290A} (lanes 1 and 6), GST-PIN1CL^{T227A S231A S290A} (lanes 2, 3, 7 and 8) or GST-PIN1CL^{T227A S231A T286A S290A} (lanes 4, 5, 9 and 10). (F) Autoradiograph (lanes 1 to 3) and coomassie stained gel (lanes 4 to 6) of an *in vitro* phosphorylation assay of his-PID (all lanes) together with his-PIN1CLsv (lanes 1 and 4) or his-PIN1CLsv^{S290A} (lanes 2 and 5) or his-PIN1CLsv^{ACT} (lanes 3 and 6).

that are fully conserved in the different PINCLs. This could be an indication that PID is a common regulator of the different PINs, which could also explain the *in vivo* effect of altered PID activity on several of these proteins (22). The strong putative PID targets are other residues than the ones identified by Nushe and co-workers (corresponding PIN1 serines-377 and -380) (38), but we provide evidence that the latter may also be modified by the PID kinase, albeit at a much reduced level. Interestingly, if the *in vivo* PID phosphor-targets in the PIN proteins do correspond to the conserved serines 290 and 231 of PIN1, this implies that multiple pathways could be involved in regulating the subcellular localization of the PIN proteins. Several phosphorylation events may also lead to different effects on PINs such as modification in the activity and/or intracellular trafficking. For example, it has been observed that the kinase PKC mediates biphasic phosphorylation of the serotonin transporter (SERT), probably leading to SERT's inactivation and subsequent internalization (24).

PIN3 provides an exception in its response to elevated levels of PID. PIN3 contains all conserved phosphorylation sites present in other PIN proteins, including the ones that are putative phosphor-substrates of PID. However, its symmetric distribution in columella cells (5) is not responsive to PID overexpression (22). Preliminary data suggest that PIN3 is sensitive to the PID induced basal-to-apical switch in polar targeting when expressed in the root epidermis (Friml and co-workers, unpublished data). This suggests that there is a cell-type specific responsiveness to PID, which may be determined by the presence of PBPs that regulate PID activity (Chapters 2

and 3 of this thesis) (30, 31). Alternatively, the unresponsiveness of PIN3 in the columella could be explained by the existence of a PID-independent mechanism that determines a “pre-polar” distribution of PINs. In this framework PID action would determine the correct pole for the PINs which are predestined to have basal-apical subcellular localization. For example, PIN1, which is affected by PID, has been shown to be still polarly distributed at the basal side of epidermal cells in the shoot apex of the *pid* mutant (22). PIN3 may not be susceptible to PID signaling in columella cells due to the hypothetical fact that it does not undergo a “pre-polar” arrangement in these cells.

To further assess the significance of the putative *in vivo* PID-mediated phosphorylation of PINs, we are currently generating *pPIN1::PIN1-GFP* constructs harboring serine to alanine or serine to aspartic acid mutations in the *in vitro* identified PID phosphor-substrates. It is our expectation that such new plant lines will report the consequences of the hypothetical reduced or constitutive PID-mediated phosphorylation for the PIN1 protein.

Hypothetical molecular mechanisms of PID-dependent PIN regulation

Striking analogies have been found between the hypothetical PID-mediated regulation of PIN protein deployment in plant cells and the sub-cellular localization of membrane proteins in animal cells. In mammals, for example, PKC has been implicated in promoting endocytosis of the serotonin and dopamine transporters as well as exocytosis of the glucose transporter GLUT4 (23-25). In another example, it has been shown that PKA phosphorylates a cytoplasmic domain of the water transporter AQP2 in order to promote its exocytosis into the apical membrane of canine renal collecting duct cells (27). The latter situation seems to be more comparable to the PID-induced PINs polar switch, since it involves direct phosphorylation of the transporter leading to its targeting to the apical cell pole. The indications that PID could be directly involved in the mediation of endo- or exocytosis in plants, as well as our unpublished observations regarding the PID substrate preference (Galvan and Offringa, unpublished data), may be significant evidence that this kinase is the plant ortholog of PKA or PKC, as previously proposed by Benjamins and co-workers (21) and Bogre and co-workers (28).

Although very interesting, these analogies still lack considerable experimental evidence regarding what exactly could be the consequences of the hypothetical PID phosphorylation of PINs *in vivo* (does it lead to endo or exocytosis?), and what are the components acting prior to or after PID action that ultimately drive PINs to their polar localization at the PM. It is known, for example, that the ARF-GEF protein GNOM is required for the polar PIN1 localization at the PM (19, 39), but the connection between PID and GNOM remains obscure. It is believed that ARF-GEFs determine the destination of membrane trafficking vesicles by specifically recruiting

vesicle-coating proteins that define their cellular location, including COP1 and clathrin coats (40). Consequently, it is possible that GNOM is required for the migration of PIN1 from endosomal compartments to the apical or basal cell pole at the PM, although not necessarily the correct one, while PID activity could be required for the localization of PIN1 in the proper cellular pole. This assumption is corroborated by observations that in *gnom* loss-of-function embryos PIN1 is at the membrane, although in randomized polarities (18), and that in epidermal cells of inflorescence apices of *pid* loss-of-function plants PIN1 is localized at basal, instead of the apical, pole in the PM (22). Considering that PID probably phosphorylates PINs *in vivo*, three situations could possibly explain the relationship between PID, PINs and ARF-GEFs such as GNOM (Figure 4). PID could phosphorylate PINs following ARF-GEF action when they are already placed at the PM, and this modification could enable PINs to stay or go to a polar position (Figure 4A). Alternatively, PID could phosphorylate PINs in endosomal compartments prior to their ARF-GEF-dependent translocation to the PM. Subsequently, upon ARF-GEF-mediated transport of PIN vesicles, the phosphorylation would be an important informative signal for the polar deposition of these proteins in the PM (Figure 4B). A third possibility is that PID counteracts an hypothetical default basal localization of PINs determined by GNOM action (Figure 4B). The relationship between GNOM and PID-mediated PIN phosphorylation needs further investigation.

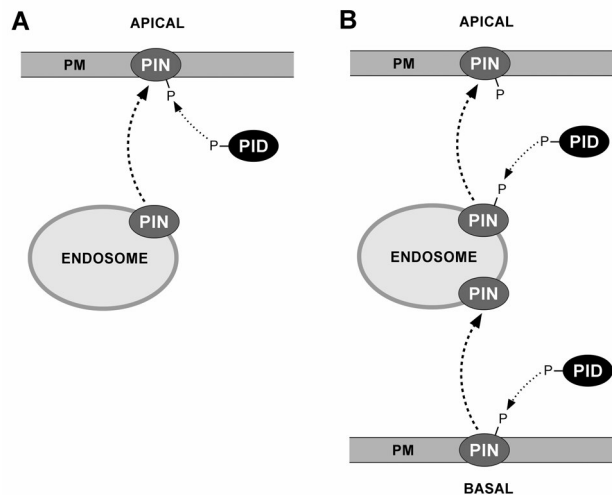


Figure 4. Models describing the molecular mechanism of PID action on PIN polar targeting. (A) PID-mediated phosphorylation of PIN proteins at the apical cell side could inhibit their endocytosis, thereby causing retention of these proteins at plasma membrane (PM). (B) PID-mediated phosphorylation of PIN proteins at the basal cell side could label these proteins for endocytosis and subsequent trafficking via endosomal compartments to the apical cell side. In parallel, PID could label endosomal PIN proteins for apical targeting.

Redundancy in PID function

Data shown in this chapter suggest that most PINs are susceptible to PID phosphorylation. However, acknowledged PID-sensitive PINs play a role in tissues such as roots (PIN1, PIN2 and PIN4) (4, 9, 32), hypocotyls (PIN1) (41), inflorescence meristem (PIN1) (3) and embryo (PIN1, PIN4) (1, 22, 32), whereas PID function is apparently limited to the latter two tissues (20-22, 42). Assuming that PID-like signaling is probably essential for PIN polarity throughout the whole plant, it is logical to assume that PID-related kinases likely regulate PINs in other tissues than in inflorescence meristems or embryos. Our previous analysis of the Arabidopsis genome identified twenty-two other members of the plant specific family of protein kinases to which PINOID belongs (21). Most likely, some of these members are also putative PINs regulators, and the comparative study of their function and activity will help to clarify their role in the regulation of the direction of PAT.

ACKNOWLEDGEMENTS

This work was funded by grant from CAPES (Brazilian Federal Agency for Post-Graduate Education, M.K.-Z.). The yeast two-hybrid plasmid pACT2-PIN1CL was kindly provided by Dr. Klaus Palme (Max-Delbruck-Laboratorium in der Max-Planck-Gesellschaft, Germany). The histidine tagged-PIN fusions pET-PIN1CLsv, pET-PIN3CL, pET-PIN4CL, pET-PIN6CL and pET-PIN7CL were kindly provided by Dr. Jiri Friml (University of Tübingen, Germany). The pGEX-PIN2CL was kindly provided by Drs. Christian Luschnig and Rene Benjamins (University of Natural Resources and Applied Life Sciences, Austria). We also thank Johan Memelink for providing pET16H, Rene Benjamins for the pGEX-PID, pAS2-PID and pACT2-PBP2 plasmids, Carlos Galvan for the plasmid encoding H-PID and for helpful comments on the manuscript, and Peter Hock for the art work.

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