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# Plasma membrane-bound AGC3 kinases phosphorylate PIN auxin carriers at TPRXS(N/S) motifs to direct apical PIN recycling

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## SUMMARY

Polar membrane cargo delivery is crucial for establishing cell polarity and for directional transport processes. In plants, polar trafficking mediates the dynamic asymmetric distribution of PIN FORMED (PIN) carriers, which drive polar cell-to-cell transport of the hormone auxin, thereby generating auxin maxima and minima that control development. The *Arabidopsis* PINOID (PID) protein kinase instructs apical PIN localization by phosphorylating PINs. Here, we identified the PID homologs WAG1 and WAG2 as new PIN polarity regulators. We show that the AGC3 kinases PID, WAG1 and WAG2, and not other plant AGC kinases, instruct recruitment of PINs into the apical recycling pathway by phosphorylating the middle serine in three conserved TPRXS(N/S) motifs within the PIN central hydrophilic loop. Our results put forward a model by which apolarly localized PID, WAG1 and WAG2 phosphorylate PINs at the plasma membrane after default non-polar PIN secretion, and trigger endocytosis-dependent apical PIN recycling. This phosphorylation-triggered apical PIN recycling competes with ARF-GEF GNOM-dependent basal recycling to promote apical PIN localization. In planta, expression domains of PID, WAG1 and WAG2 correlate with apical localization of PINs in those cell types, indicating the importance of these kinases for apical PIN localization. Our data show that by directing polar PIN localization and PIN-mediated polar auxin transport, the three AGC3 kinases redundantly regulate cotyledon development, root meristem size and gravitropic response, indicating their involvement in both programmed and adaptive plant development.

**KEY WORDS: Polar auxin transport, PIN efflux carrier, PID, WAG1, WAG2, Basal and apical recycling, Polar trafficking, Transcytosis, Endocytosis-related Rab5 pathway, *Arabidopsis***

## INTRODUCTION

The major plant signaling molecule auxin (indole-3-acetic acid or IAA) directs numerous developmental processes through its polar cell-to-cell transport-generated maxima and minima that regulate cell division, differentiation and growth. Polar auxin transport (PAT) involves at least three types of transporter proteins, of which the PIN-FORMED (PIN) auxin efflux carriers are key drivers as they determine the direction of transport through their asymmetric subcellular localization at the plasma membrane (PM) (Benjamins and Scheres, 2008; Sorefan et al., 2009; Tanaka et al., 2006).

Previously, the PINOID (PID) protein serine/threonine kinase has been identified as a regulator or PAT (Benjamins et al., 2001), and it was shown that PID is a PM-associated kinase that directs targeting of PIN proteins to the apical (shootward) side of the PM by phosphorylating these transporters in their large central hydrophylic loop (PINHL) (Friml et al., 2004; Michniewicz et al., 2007). However, the site of PID action and the biochemical and cellular mechanisms by which PID promotes apical PIN polarity have remained largely unresolved. Basal (rootward) PIN polarity generation involves non-polar PIN secretion followed by clathrin-mediated PIN endocytosis (Dhonukshe et al., 2007) and ARF-GEF GNOM-dependent (Geldner et al., 2003) basal PIN endocytic recycling (Dhonukshe et al., 2008). Based on this, PID could generate apical PIN localization in three different ways: (1) PID modifies newly synthesized PIN at the Golgi for its apical secretion; (2) PID modifies PIN at endosomes for its apical recycling; or (3) PID modifies PIN at the PM to promote apical recycling following endocytosis. Moreover, whereas all *pid* mutants develop pin-like inflorescences, correlating with basal PIN1 localization (Friml et al., 2004), the three-cotyledon phenotype is not fully penetrant, even in strong *pid* alleles for which PIN1 localization in embryo epidermis cells is either basal or apical (Treml et al., 2005). In addition, apical cargo such as PIN2 in root epidermis cells remains apical in *pid* alleles (Sukumar et al., 2009). In view of the key role for PID in PIN polar targeting, these observations strongly suggest that there are other protein kinases that act redundantly with PID in establishing PIN polarity.

By testing representative members of the AGC kinase family to which PID belongs, here we identified WAG1 and WAG2 as new PIN polarity-mediating kinases. Our result show that the central serines in the three conserved TPRXS(N/S) motifs in the PINHL are the key residues on which PID, WAG1 and WAG2 act, to regulate programmed embryo development and adaptive root development. Our detailed dynamic imaging analysis puts forward a model by which PID, WAG1 and WAG2 phosphorylate PINs

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predominantly at the PM, thereby instructing endocytosis-dependent recruitment of these PINs to the GNOM-competitive apical recycling pathway for apical PIN polarity generation.

## MATERIALS AND METHODS

### Plant lines and growth conditions

The *Arabidopsis* lines *pid-14* (SALK_049736), *wag1* (SALK_002056), *wag2* (SALK_070240) (Cheng et al., 2008; Santner and Watson, 2006), *eir1-1* (Luschnig et al., 1998), *35S::PID* (Benjamins et al., 2001), *PID::PID-VENUS* (Michniewicz et al., 2007), *gnom* (Geldner et al., 2004), *snx1* (Jaillais et al., 2006), *vps29* (Jaillais et al., 2007) and *cpi1* (Men et al., 2008) have been described previously. Genotyping and plant growth was performed as described (Huang et al., 2010). Gene-specific primers are listed in Table S1 in the supplementary material.

### Constructs, molecular cloning, plant transformation and protein biochemistry

The constructs *pGEX-PID* (Axelos et al., 1992) and *pGEX-PIN2HL* (Abas et al., 2006) and all T-DNA constructs (see below) were introduced into *Agrobacterium tumefaciens* strain C58C1 (GV3101) (Van et al., 1974) or AGL1 (Lazo et al., 1991) by electroporation. Plant transformation was performed in *Arabidopsis* Columbia ecotype (Col-0), the *eir1-1* mutant or the *pid wag1 wag2* triple mutant using the floral-dip method (Clough and Bent, 1998). Protein purification and in vitro phosphorylation assays were performed as described previously (Huang et al., 2010).

### Molecular cloning

Primers used for cloning are listed in Table S1 in the supplementary material. The coding region of *PID* was amplified from *Arabidopsis thaliana* ecotype Columbia (Col-0) cDNA from siliques using primer set PID attB F and PID-Stop attB R. The genomic clones for *WAG1* and *WAG2* comprising, respectively, 3205 bp and 3402 bp upstream from the ATG, were amplified from *Arabidopsis thaliana* Col-0 genomic DNA using primer sets: attB1 WAG1pWAG1 3 F and WAG1-Stop attB R; attB1 WAG2p 2 F and WAG2-Stop attB R. Coding regions for *WAG* genes were PCR-amplified from *Arabidopsis thaliana* Col-0 genomic DNA using primer sets: WAG1 attB F and WAG1-Stop attB R; WAG2 attB F and WAG2-Stop attB R.

For constitutive overexpression, the Gateway recombination cassette was inserted in frame with the *YFP*, *CFP* or *mRFP1* coding region between the *CaMV 35S* promoter and the *CaMV 35S* terminator of *pART7* (Gleave, 1992). For the *pGEX*-based destination vector, the Gateway recombination cassette was inserted in frame with the *GST* coding region. For the *pGreenII*-based destination vector, the recombination cassette was inserted in frame with the *YFP* coding region and the *CaMV 35S* terminator into *pGreenII0179* (Hellens et al., 2000). Expression vectors *pGEX-WAG1*, *pGEX-WAG2*, *pART7-PID-mRFP*, *pART7-WAG1-YFP* and *pART7-WAG2-CFP* were constructed using the Gateway technology (Invitrogen). Overexpression cassettes containing the genes of interest were digested with *Not*I and cloned into *pGreenII* binary vectors (Hellens et al., 2000) for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. The *pPZP221-PIN2::PIN2-VENUS* construct was kindly provided by Christian Luschnig (BOKU, Vienna, Austria). For the site-directed mutagenesis, we used the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene). The oligonucleotides used to introduce mutations in the PIN2HL cDNA and the *pPZP221-PIN2::PIN2-VENUS* construct are listed in Table S1 in the supplementary material.

For the AGC-kinase-inducible overexpression studies, a modified version of *pER8* was used (Zuo et al., 2000). Promoters, coding regions and terminators were cloned into, respectively, the first, second and third box of the Multisite Gateway Cassette (Invitrogen) that was introduced into the modified *pER8* vector. Details of the inducible system will be described elsewhere (A.P.M. and B.S., unpublished). Three different promoters were used in the inducible system and in the constitutive expression lines: ubiquitously expressing 243 bp *G10-90* promoter (Zuo et al., 2000), cortex-specific 550 bp *CO2* promoter (Heidstra et al., 2004) and epidermis-specific 2472 bp *WER* (*WEREWOLF*) promoter (Lee and Schiefelbein, 1999). *PINOID*, *WAG1* and *WAG2* cDNAs were amplified by using primers shown in Table S1 in the supplementary material. *PIN1-GFP2* was amplified from *PIN1::PIN1-GFP2* plasmid (Xu et al., 2006) by using primers shown in Table S1 in the supplementary material. Amplified *PID*, *WAG1*, *WAG2* and *PIN1-GFP2* were recombined into a *pDONR* vector according to the manufacturer's protocol (Invitrogen) to obtain *G10-90::XVE>>PINOID-YFP*, *G10-90::XVE>>PINOID*, *G10-90::XVE>>WAG2*, *G10-90::XVE>>WAG1*, *WER::XVE>>PIN1-GFP2*, *WER::XVE>>PINOID-mCherry*, *WER::XVE>>PINOID*, *CO2::XVE>>PINOID-YFP* and *CO2:: PINOID-YFP* gene cassettes.

*WAG1::ERGFP* and *WAG2::ERGFP* were generated by fusing 2 kb of upstream sequences from *WAG1* and *WAG2* with an *ERGFP* reporter gene engineered in pGREENII-0229. *WAG1::WAG1-GFP* and *WAG2::WAG2-GFP* gene fusions were generated by fusing 3.5 kb of genomic fragments (including promoter, exon and intron regions) of *WAG1* and *WAG2* to the N-terminal of *EGFP* reporter gene engineered in pGREENII-0229. *PIN2::PIN2-mCherry* was generated by replacing the *GFP* fragment of the *PIN2::PIN2-GFP* construct (Xu and Scheres, 2005) with *mCherry*.

*PIN1-GFP2* was excised from *PIN2::PIN1-GFP2* and cloned into vector *pBluescript*. *GFP* was replaced with *EosFP* to have *PIN1-EosFP2*. *PIN1-EosFP2* was PCR amplified with 221PIN1F and 221PIN1R to bring it into second box of Multisite Gateway. *PIN2-EosFP* cloning has been previously described (Dhonukshe et al., 2007). *PIN2-EosFP* was PCR-amplified from vector *pBluescript* by using primers *221PIN2F* and *221PIN2R* to bring it into second box of Multisite Gateway. *PIN2* promoter was amplified from *Arabidopsis* genomic DNA by using primers *pPIN2F* and *pPIN2R* and cloned into the first box of Multisite Gateway. After that, *PIN2::PIN1-EosFP2*, *PIN2::PIN2-EosFP*, *WER::XVE>>PIN1-EosFP2*, *WER::XVE>>PIN2-EosFP* were assembled in the Multisite Gateway system. The primer sequences are shown in Table S1 in the supplementary material.

*mRFP-DNAra7* was generated from *GFP-DNAra7* (Dhonukshe et al., 2006) by replacing the *GFP* with the *mRFP* coding region. *mRFP-DNAra7* was PCR-amplified with primers *221mRFPF* and *221Ara7R* to bring into the second box of Multisite Gateway and thereafter the gene cassettes *CO2::XVE>>DN-Ara7-mRFP*, *CO2::DN-Ara7-mRFP WER::XVE>>DN-Ara7-mRFP* and *WER:: DN-Ara7-mRFP* were assembled.

### Drugs

Estradiol (Sigma) and BFA (Molecular Probes) were used from DMSO stock solutions at 5 μM estradiol and 50 μM BFA working concentration for indicated periods.

### Immunolocalization

Wholemount immunolocalizations were performed on 3- to 5-day-old seedlings fixed in 4% paraformaldehyde in MTSB buffer as described previously (Friml et al., 2003) using an InSituPro robot (INTAVIS, Cologne, Germany). Rabbit anti-PIN1 (Friml et al., 2004), anti-PIN2 (Abas et al., 2006) and anti-PIN4 (Friml et al., 2002) primary antibodies (1/200) and Alexa (1/200, Molecular Probes) or Cy-3- (1/600, Dianova) conjugated anti-rabbit secondary antibodies were used for detection.

### Confocal microscopy

Immunofluorescence and live cell confocal laser-scanning microscopy were performed as described (Dhonukshe et al., 2006; Dhonukshe et al., 2007; Friml et al., 2004). The images were processed by ImageJ (http://rsb.info.nih.gov/ij/) and assembled.

### Accession numbers

The *Arabidopsis* Genome Initiative locus identifiers for the genes mentioned in this manuscript are as follows: *PID* (At2g34650), *WAG1* (At1g53700), *WAG2* (At3g14370), *AGC3-4* (At2g26700), *AGC1-1* (At5g55910), *AGC1-2* (At4g26610), *PK5* (At5g47750), *PK7* (At3g27580), *AGC2-1* (At3g25250), *AGC2-2* (At4g31000), *AGC2-3* (At1g51170), *PIN1* (At1g73590), *PIN2* (At5g57090) and *GNOM* (At1g13980).

## RESULTS

### PID and WAG protein kinases redundantly instruct PIN polarity during cotyledon and root development

PID and the WAG kinases belong to the plant-specific AGCVIII family of kinases, within which they cluster into the AGC3 subfamily (see Fig. S1 in the supplementary material). To analyze whether PID-related AGC3 kinases WAG1 and WAG2 (see Fig. S1 in the supplementary material) act redundantly with PID in regulating PIN polarity, we used the previously described *pid-14*, *wag1* and *wag2* loss-of-function mutant alleles (Cheng et al., 2008; Santner and Watson, 2006) to generate double- and triple-mutant combinations. Of the *pid-14* mutant seedlings 47% developed three cotyledons (Fig. 1A,F), consistent with previous observations for other complete loss-of-function *pid* alleles (Benjamins et al., 2001; Bennett et al., 1995; Christensen et al., 2000). In the *pid wag1* or *pid wag2* double mutants, the penetrance for the cotyledon defects remained ~50%, but a significant number of seedlings developed only one cotyledon or even lacked cotyledons (Fig. 1B,C,F). This no-cotyledon phenotype was fully penetrant for the *pid wag1 wag2* triple mutant: among 99 progeny of a *pid+ wag1 wag2* plant, 19 lacked cotyledons and were genotyped as *pid wag1 wag2* triple homozygous mutants (Fig. 1D-F), whereas the remaining 80 seedlings developed two cotyledons and were genotyped as *pid-14+ wag1 wag2* (*n*=53) or *wag1 wag2* (*n*=27). The no-cotyledon phenotype has also been observed for *pid pin1* double loss-of-function mutants (Furutani et al., 2007), suggesting that the three AGC3 kinases act redundantly on PIN1 and that their activity is crucial for proper cotyledon development. Immunolocalization showed that PIN1 polarity was predominantly basal with some lateral localization in epidermal cells of triple mutant embryos (Fig. 1I,J), whereas it was apical in *wag1 wag2* embryos (Fig. 1G,H), and apical in some and basal in other epidermal cells of *pid* mutant embryos (Treml et al., 2005). This corroborates the redundant action of the three AGC3 kinases on apical PIN1 polarization in the embryo, which is essential for proper initiation and development of cotyledons. Our results are largely in line with the genetic data by Cheng and coworkers (Cheng et al., 2008), except that in our hands the no-cotyledon phenotype was already fully penetrant for the *pid wag1 wag2* triple mutant.

In our analysis, the *pid wag1* and *pid wag2* double mutant roots showed strong waving phenotypes, like *wag1 wag2* double mutant roots (Fig. 1B,C,N) (Santner and Watson, 2006). Unlike the double mutants, however, the roots of *pid wag1 wag2* triple mutant seedlings were clearly agravitropic (Fig. 1D,N) and significantly shorter than those of wild-type (WT) seedlings (Fig. 1K-M). The auxin reporter *DR5rev::ER-GFP* (*DR5::GFP*) was expressed more strongly on both sides of *pid wag1 wag2* root tips compared with WT (Fig. 1P,O), indicating higher auxin levels that inhibit root growth and thus explaining the short-root phenotype of *pid wag1 wag2*. Moreover, this *DR5::GFP* expression persisted after gravity stimulation (Fig. 1R,Q), indicating that the agravitropy of triple mutant roots is due to their inability to asymmetrically distribute auxin (Fig. 1Q) and suggesting defects in PIN polarity. Indeed, the most distal cells of the triple-mutant root epidermis displayed basal localization of PIN2 as compared with its apical localization in WT, and the clear apical PIN2 polarity observed in *wag1 wag2* lateral root cap cells was absent in triple-mutant roots. Basal localization of PIN2 in the cortex was not affected in *pid wag1 wag2* (Fig. 1T,S). Our expression analysis (Fig. 1U-Y), together with previous expression studies (Cheng et al., 2008; Michniewicz et al., 2007; Santner and Watson, 2006), indicate that PID, WAG1

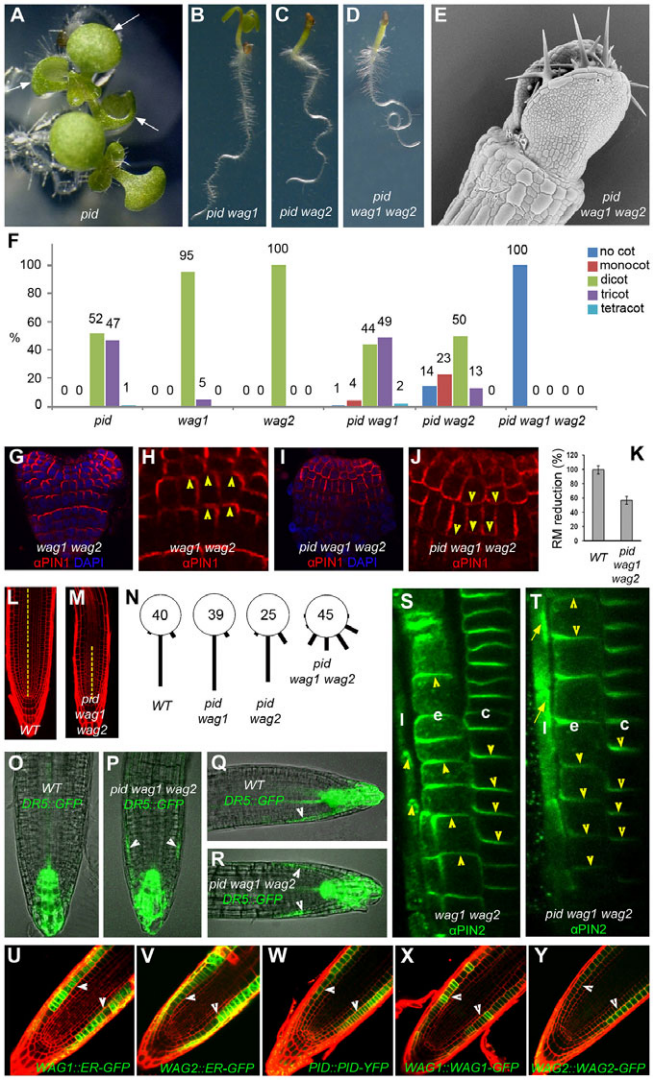


**Fig. 1. PID and WAG protein kinases redundantly instruct apical PIN polarity during cotyledon and root development.** (**A-F**) Cotyledon phenotypes. (A) A three-cotyledon seedling (white arrows) in a *pid*+ segregating population. (B) A tricot *pid wag1* seedling. (C) A no-cot *pid wag2* seedling. (D) A no-cot *pid wag1 wag2* seedling showing agravitropic root growth. (E) Scanning electron microscopy detail of the apex of a no-cot *pid wag1 wag2* seedling. (F) Frequency of cotyledon defects observed in the indicated mutant lines, assuming that 1 in 4 seedlings is homozygous for the *pid* mutation. About 400 seedlings were scored for each line. (**G-J**) Wholemount immunolocalization of PIN1 in *wag1 wag2* (G,H) or *pid wag1 wag2* (I,J) embryos. (G,I) Merge of the PIN1 (Cy-3) and the 4′-6-diamidino-2-phenylindole (DAPI) images. (H,J) Magnified section of the PIN1 (Cy-3) image**.** Yellow arrowheads depict PIN polarity. (**K-M**) The root meristem of *pid wag1 wag2* triple mutants is shorter than wild type (WT). (**N**) Root gravitropic response histogram of the indicated lines. The number of seedlings scored per line is indicated in the middle of each circle. (**O-R**) Expression of the auxin reporter *DR5rev::ER-GFP* in wild-type (WT) and *pid wag1 wag2* triple-mutant roots before (O,P) and after (Q,R) gravity stimulation. White arrowheads indicate enhanced *DR5rev::ER-GFP* expression in the lateral root cap. (**S**,**T**) Wholemount immunolocalization of PIN2 in roots of 5-day-old *wag1 wag2* (S) or *pid wag1 wag2* (T) seedlings. c, cortex; e, epidermis; l, lateral root cap. Yellow arrowheads depict PIN polarity. (**U-Y**) Expression of *WAG1* and *WAG2* promoter *ER-GFP* fusions (U,V) or *PID* gene *VENUS* (W) or *WAG1*- or *WAG2* gene *GFP* fusions (X,Y) in *Arabidopsis* roots. White arrowheads point to the expression in the epidermis and lateral root cap.

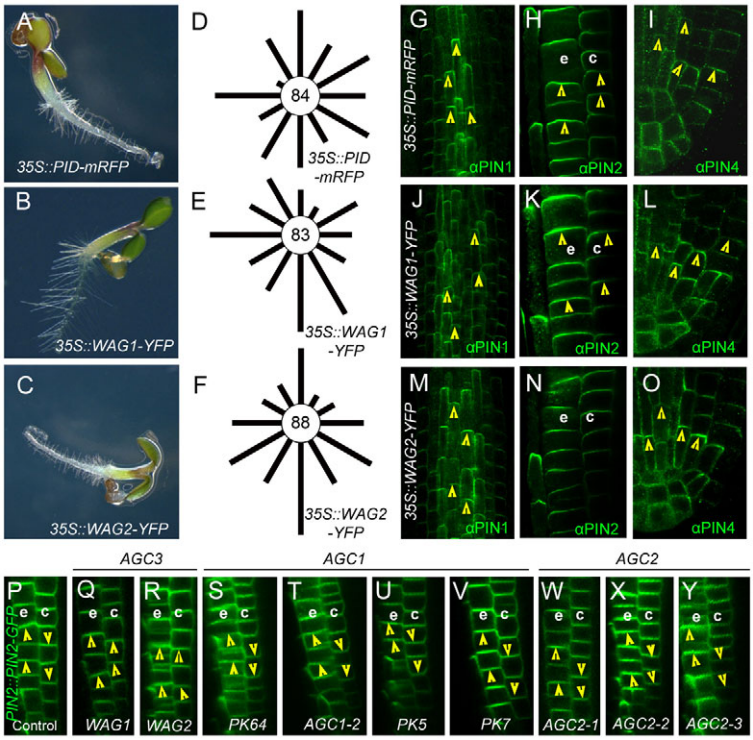


**Fig. 2. Overexpression of PID, WAG1 and WAG2, but not of other AGC kinases, instructs apical localization of PIN proteins.** (**A-O**) Overexpression of PID-mRFP, WAG1-YFP or WAG2-YFP leads to comparable seedling phenotypes, including root meristem collapse (A-C) and agravitropic root growth (D-F) as a result of apically localized PIN1 in the root stele (G,J,M), PIN2 in the cortex (H,K,N) and PIN4 in the root meristem (I,L,O). PIN proteins were detected by wholemount immunolocalization. (**P-Y**) Estrogen-inducible expression of WAG1 and WAG2, but not of other AGC kinases, leads to apicalized PIN2-GFP localization in the root cortex. c, root cortex; e, root epidermis. Yellow arrowheads depict PIN polarity.

and WAG2 are expressed predominantly in the epidermis and in the lateral root cap, and that their loss-of-function specifically perturbs apical PIN localization and disrupts upward auxin flow in these layers, leading to defects in cotyledon development, root growth and root gravitropism.

## Overexpression of PID, WAG1 and WAG2, but not other ACG kinases induces basal-to-apical PIN polarity shifts

We next analyzed the effect of overexpression of these kinases on PIN polarity and plant development. Seedlings of a *35S::PID-mRFP1* control line showed phenotypes previously observed for *PID* overexpression (Benjamins et al., 2001), such as agravitropic growth and eventual collapse of the main root meristem (Fig. 2A,D). Overexpression of WAG1-YFP and WAG2-YFP resulted in similar phenotypes (Fig. 2B,C,E,F). Similarly, estradiol-inducible overexpression of PID, WAG1 or WAG2 led to gravity loss and collapse of the main root meristem preceded by loss of the auxin maximum, as reported by the disappearance of *DR5::GFP* expression (see Fig. S2A-Q in the supplementary material). This was further supported by significant reduction in the root meristem collapse by NPA-mediated auxin efflux inhibition (Fig. S2Q). Immunolocalization showed that PIN1, PIN2 and PIN4 were all apicalized by PID, WAG1 or WAG2 overexpression (Fig. 2G-O; see Fig. S3A-H in the supplementary material). These results confirm our observations in *pid wag1 wag2* loss-of-function embryos and roots that the redundant activity of these kinases triggers apical PIN polarization, and raised the question whether other plant AGC kinases are capable of switching PIN polarity. By using the estradiol-inducible expression system we tested the effect of overexpression of AGC1-1 [also referred to as PK64 (Mizoguchi et al., 1992) or D6K (Zourelidou et al., 2009)], AGC1-2, PK5 and PK7 as representatives of the AGC1 subfamily on PIN2-GFP polarity, and also tested three AGC2 kinases. Only WAG1 and WAG2 induced the basal to apical shift of PIN2-GFP in root cortex cells, eventually leading to root meristem collapse, whereas the other kinases tested did not affect PIN2-GFP polarity (Fig. 2P-Y) or root meristem integrity (data not shown). These results indicate that, from the tested AGCVIII kinases, PID, WAG1 and WAG2 are the PIN polarity regulators.

## Phosphorylation of conserved serines in the PIN2HL by PID, WAG1 and WAG2 is essential for auxin dynamics during gravitropic root growth

Previously, we showed that PID phosphorylates the PIN2HL in vitro (Michniewicz et al., 2007) and our current analysis showed that apical PIN2 localization in root epidermis and the lateral root cap matches with the epidermis and later root cap-specific predominant expression of PID, WAG1 and WAG2 (this study). Therefore, we sought to identify the PID, WAG1 and WAG2 phosphorylated residues in the PIN2HL. Based on our recent identification of the middle serine residue in three conserved TPRXS(N/S) motifs in the PIN1HL as PID targets, and the conservation of these motifs between PIN proteins (Huang et al., 2010), we first tested whether these serines in the PIN2HL (at positions 237, 258 and 310, renumbered hereafter as 1, 2 and 3) were also phosphorylated by PID (Fig. 3A,B). For this we performed in vitro phosphorylation assays using mutant PIN2HL versions in which the three serines (S) were replaced by alanines (A). In vitro incubation of the S1,2,3,A mutant version with GST-PID showed that these three serines are the only PID phospho-targets in the PIN2HL (Fig. 3B). Similar results were obtained when the WT and mutant GST-PIN2HL versions were incubated with purified GST-tagged WAG1 and WAG2 (Fig. 3C). The results reveal that the three AGC3 kinases phosphorylate the PIN2HL in vitro and that the substrate-specificity among the three kinases is conserved, thereby corroborating the redundant action of PID, WAG1 and WAG2 to promote apical PIN polarity.

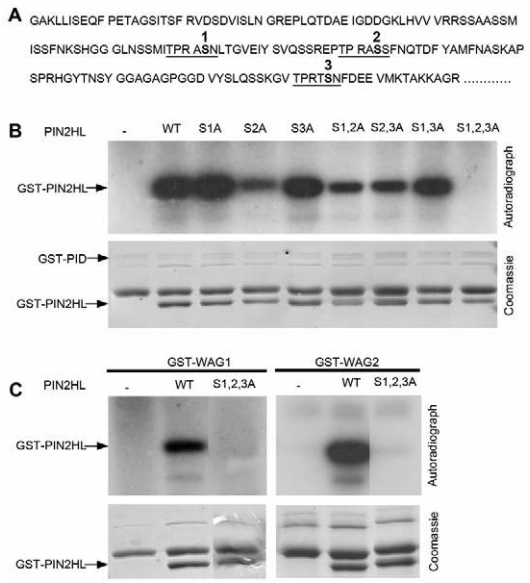


**Fig. 3. The central serines in three conserved TPRXS(N/S) motifs of the PIN2HL are the phosphorylation targets of PID, WAG1 and WAG2 kinases in vitro.** (**A**) N-terminal part of the PIN2HL with the phosphoserines in the three TPRXS(N/S) motifs indicated. (**B**) GST-PIN2HL is phosphorylated by GST-PID. The phosphorylation is gradually reduced when one or two serines are replaced with alanines (indicated as S1A, S2A, S3A, S1,2A, S2,3A and S1,3A, respectively) and is completely abolished when all of the three serines are mutated to alanines (S1,2,3A). (**C**) GST-PIN2HL, but not the GST-PIN2HL S1,2,3A mutant form, is phosphorylated by GST-WAG1 or GST-WAG2.

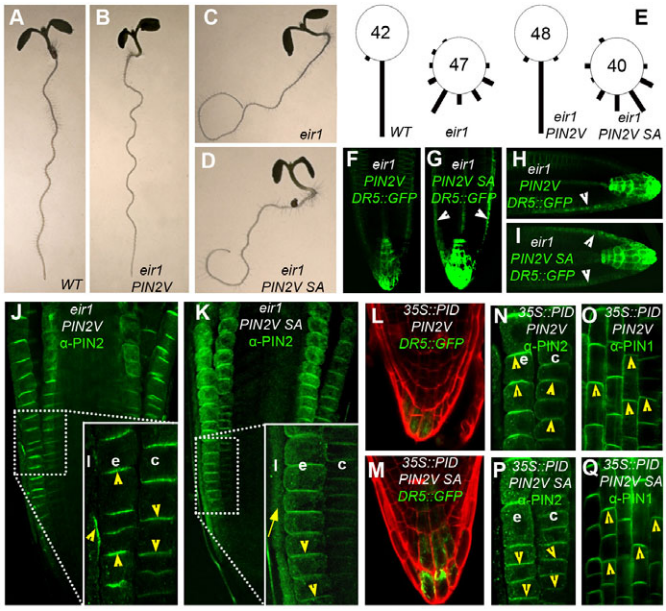


**Fig. 4. AGC3 kinase-mediated phosphorylation of PIN2 controls apical PIN2 localization, auxin distribution and root gravitropism.** (**A-D**) Phenotype of 7-day-old seedlings of Columbia wild-type *Arabidopsis* (A), *eir1-1 PIN2::PIN2V* (B), *eir1-1* (C) and *eir1-1 PIN2::PIN2V SA* (D). (**E**) Root gravitropic response histogram of the indicated lines. The number of seedlings scored per line is indicated in the middle of each circle. (**F-I**) Expression of the *DR5rev::ER-GFP* reporter in *eir1-1 PIN2::PIN2V* (F,H) and *eir1-1 PIN2::PIN2V SA* (G,I) seedlings roots before (F,G) and 48 hours after (H,I) gravity stimulation. White arrowheads indicate enhanced *DR5rev::ER-GFP* expression. (**J**,**K**) Immunolocalization of PIN2 in roots of 5-day-old seedlings of the *eir1-1 PIN2::PIN2V* (J) and *eir1-1 PIN2::PIN2V SA* (K) lines. Yellow arrow in the magnified image indicates loss of PIN2 polarity in the lateral root cap. Yellow arrowheads depict PIN polarity. c, cortex; e, epidermis; l, lateral root cap. (**L**,**M**) *DR5rev::ER-GFP* expression is higher in *35S::PID PIN2::PIN2V SA* (M) than in *35S::PID PIN2::PIN2V* (L). (**N-Q**) Immunolocalization of PIN2 (N,P) and PIN1 (O,Q) in 3-day-old *35S::PID PIN2::PIN2V* (N,O) or *35S::PID PIN2::PIN2V SA* (P,Q) seedling roots. Yellow arrowheads depict PIN polarity.

To investigate the biological significance of the AGC3 kinase-dependent phosphorylation for PIN2 in plants, mutations were introduced into a *PIN2::PIN2-Venus* construct to replace all three serines (S) by alanines (A). The resulting *PIN2::PIN2-Venus S1,2,3A* loss-of-phosphorylation mutant construct and the WT *PIN2::PIN2-Venus* construct (hereafter named *PIN2V SA* and *PIN2V*, respectively) were transformed into the *Arabidopsis pin2* loss-of-function allele *eir1-1*, and fluorescence-positive, single-locus T-DNA insertion lines were selected for further analysis. *PIN2V* seedling roots showed wild-type wavy and gravitropic growth (Fig. 4A,B,E). By contrast, the *eir1-1* and *PIN2V SA* seedling roots exhibited a linear growth pattern interrupted by random turns (Fig. 4C-E). These results indicate that AGC3 kinase-mediated phosphorylation of PIN2 is important for normal wavy, gravitropic root growth. For *pin2* loss-of-function mutants, the agravitropic root growth has been correlated with changes in the auxin dynamics in the root tip (Abas et al., 2006; Ottenschlager et al., 2003). Observation of the *DR5::GFP* auxin reporter expression showed that in *PIN2V SA* seedling roots the *DR5::GFP* signal is stronger on both sides of the root compared with *PIN2V* (Fig. 4F,G). Following gravity stimulation, the *DR5::GFP* signal in *PIN2V SA* roots remained symmetrically on both sides of root meristem (Fig. 4I), which was in contrast to the asymmetric signal observed in *PIN2V* roots (Fig. 4H). A similar distribution was observed in *pid wag1 wag2* mutant roots (Fig. 1P,R). The similarities in root phenotypes between the *PIN2V SA* and the *pid wag1 wag2* triple mutant seedlings corroborate that the three serines are the phosphorylation targets of the PID, WAG1 and WAG2 kinases in vivo, and indicate that AGC3 kinase activity in the root channels through PIN2 to facilitate normal root growth. To correlate the phenotypes and changes in auxin dynamics in the *PIN2V SA* and *pid wag1 wag2* mutant roots with changes in PIN2 polarity, we observed *PIN2V SA* directly by confocal microscopy or following wholemount immunolocalization using PIN2-specific antibodies. In agreement with previous observations for PIN2 or PIN2-GFP (Abas et al., 2006; Muller et al., 1998), PIN2V was apically localized in lateral root cap (LRC) and epidermal cells, and showed basal localization in young cortical cells (Fig. 4J) and a basal-to-apical shift in older cortical cells. By contrast, PIN2V SA was basally localized in young epidermis and cortex cells of the distal root tip, whereas older epidermis and cortex cells showed a gradual basal-to-apical shift (Fig. 4K), a PIN2 localization pattern similar to that in *pid wag1 wag2* triple-mutant roots.

*PIN2V SA* expression in the *eir1-1 35S::PID* background resulted in a stronger *DR5::GFP* signal in the collumella compared with *PIN2V* (Fig. 4M,L). Whereas PIN2V polarity in cortical root cells was shifted from basal-to-apical by *PID* overexpression, *PIN2V SA* polarity remained predominantly basal as in the WT background (Fig. 4P,N), demonstrating that the loss-of-phosphorylation protein was insensitive to kinase overexpression. In the stele cells of the same roots, PIN1 was localized at the apical

side (Fig. 4O,Q), demonstrating that *PID* overexpression in these seedlings was sufficient to induce a basal-to-apical shift in PIN polarity.

These results are in line with the above observations on the redundant role of these three AGC3 kinases in instructing apical PIN localization by phosphorylating the three TPRXS(N/S) motifs within PIN, but also indicate that in some cells, such as older epidermal and cortex cells, PIN apicalization probably involves an AGC3 kinase-unrelated mechanism.

## PID, WAG1 and WAG2 instruct apical PIN polarity through their non-polar PM association

As the root provides a relatively easier PIN polarity-based response system in which individual cells are traceable, we focused our further analysis on roots. Previous immunolocalization of PID-VENUS (YFP) in root epidermis cells indicated that PID is a PM-associated protein kinase that localizes predominantly at the apical and basal cell sides, where it partially colocalizes with its PIN phosphorylation targets (Michniewicz et al., 2007). More detailed analysis of the *PID::PID-YFP* line (Michniewicz et al., 2007) and our *WAG1::WAG1-GFP* and *WAG2::WAG2-GFP* lines showed that, in their epidermal expression domain, all the three fusion proteins also display a significant signal at the lateral PM. As the slightly stronger signal at the apical and basal PM can be explained by overlapping signals from neighboring cells, we conclude that the kinases localize symmetrically at the PM (Fig. 5A-C) in the same cells where PIN2 shows polar localization (Fig. 5E), generating a situation in which a kinase is non-polar and its substrate polar.

To address the question at which location in the cell these kinases phosphorylate PINs, we focused our analysis on PID. Inducible expression of *PID* showed that newly synthesized PID arrives at the PM in a non-polar manner (Fig. 5D) and that PID retains its symmetric localization after shifting PIN1 localization from basal to apical (Fig. 5F). Apart from their PM localization, PID, WAG1 and WAG2 also localized to subcellular punctuate structures (Fig. 5A-C), and more detailed analysis of the *PID::PID-YFP* line showed that PID only partially colocalizes with the endocytic tracer FM-4-64 (Fig. 5G). Moreover, PID colocalizes with PIN1 predominantly at the PM and much less on the intracellular vesicles in *Arabidopsis* root cells (Fig. 5H,I), similar to what has previously been observed in *Arabidopsis* protoplasts (Furutani et al., 2007), indicating that PIN1 and PID reside on divergent intracellular vesicles. Intriguingly, in the *cpi1* sterol biosynthesis mutant (Men et al., 2008), arrival of newly synthesized PID at the PM was reduced after a few hours of steroid-induced kinase expression as compared with the WT (Fig. 5J,K) and remained less effective in provoking rapid basal-to-apical PIN localization (Fig. 5L,M) and the root phenotypes associated with it (Fig. 5N). Based on the pleiotropic phenotypes of *cpi1-1* and the necessity of sterols for many processes, we cannot exclude that other processes in the *cpi1-1* mutant background are indirectly contributing to the observed defects in PIN1 trafficking. Nonetheless, the results showing (1) non-polar PM arrival of newly synthesized PID, (2) colocalization of PID and PIN at the PM and only occasionally in intracellular vesicles, (3) PIN relocation after PID arrival at the PM, (4) persistence of non-polar PID after PIN relocation and (5) the reduced PM localization of PID in the *cpi1* mutant background ameliorating its effect on PIN polarity, all put forward a model in which PID phosphorylates PINs mainly at the PM to induce their apicalization. As WAG1 and WAG2 also localize non-polarly at the PM similar to PID, and act redundantly with PID to regulate PIN polarity, we consider that WAG1 and WAG2 also exert their effect by phosphorylating PIN mainly at the PM.

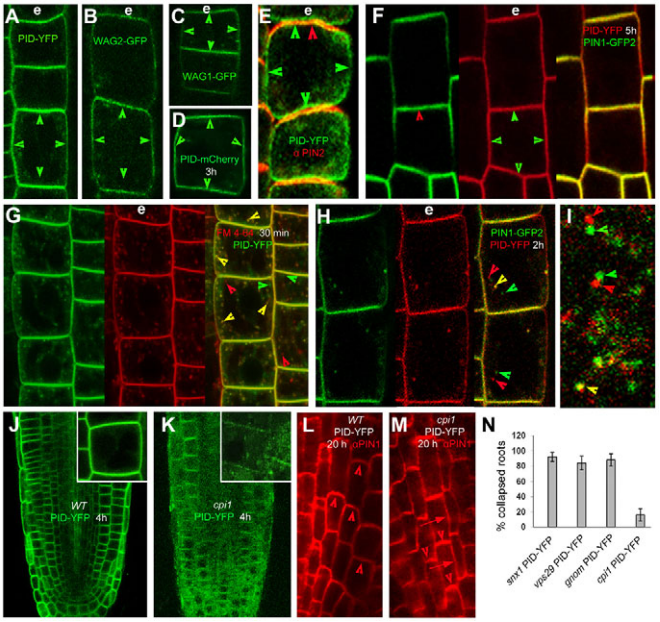


**Fig. 5. PID, WAG1 and WAG2 predominantly phosphorylate PINs at the PM to induce their apical trafficking.** (**A-C**) Predominant non-polar PM localization of *PID::PID-YFP* (A), *WAG1::WAG1-GFP* (B) and *WAG2::WAG2-GFP* (C). (**D**) Non-polar arrival of PID at the PM following 3 hours of induced expression of *WER::XVE>>PID-mCherry*. (**E**) Apical PIN2 and non-polar PID localization at the PM of an epidermis cell. (**F**) Apical *PIN2::PIN1-GFP2* (green) and non-polar PID-YFP (red) localization after 5 hours of induction of *G10-90::XVE>>PID-YFP* expression. Green and red arrowheads indicate PID and PIN2 PM localization, respectively. (**G**) PID-YFP (green arrowheads) shows partial colocalization with the endocytic tracer FM4-64 (red arrowheads). (**H**,**I**) PID-YFP (red arrowheads) and PIN1-GFP2 (green arrowheads) predominantly colocalize (yellow arrowheads) at the PM (H, 44% of PID with PIN1, and 83% of PIN1 with PID) and only marginally at intracellular vesicles (I, 5% of PID with PIN1, and 4% of PIN1 with PID). (**J**,**K**) After 4 hours of *G10-90::XVE>>PID-YFP* expression, *cpi1* sterol mutant roots (K) show much less PM localization of PID-YFP than WT roots (J). The same confocal settings were used for panels J and K. Insets show higher magnification views. (**L-N**) After 20 hours of *G10-90::XVE>>PID-YFP* expression, the apical-to-basal switch in PIN1 polarity is observed in wild-type (L), but not *cpi1*, roots (M). As a result, the PID overexpression-induced root meristem collapse is delayed in 6-day-old seedlings of *cpi*, but not of *snx1*, *vps29* or *gnom* mutant background (N). Red arrowheads indicate apical PIN localization (in L) and basal PIN localization (in M), and red arrows indicate lateral PIN1 localization (in M).

## Redundant PID and WAG action recruits PINs to an ARF-GEF GNOM-competitive apical recycling pathway

To probe whether the action of the PID and WAG kinases on PIN polarity involves known PIN trafficking regulators, such as GNOM (Geldner et al., 2003; Steinmann et al., 1999), SNX1 (Jaillais et al., 2006) and VPS29 (Jaillais et al., 2007), we tested the effect of induced PID expression on PIN polarity in the *snx1*, *vps29* and *gnom* mutant backgrounds. In all three mutants, PID arrived at the PM as in the WT control and triggered PIN1 and PIN2 apicalization, leading to the root meristem collapse (see Fig. S4A-L in the supplementary material). These results show that kinase-induced apical PIN polarity does not act through SNX1, VPS29 and GNOM. Interestingly, in *gnom*, the kinase-induced PIN

polarity switch occurred slightly faster than in WT, suggesting that PID and GNOM operate in two opposing pathways for regulating PIN polarity.

We used the fungal toxin brefeldin A (BFA), which targets GNOM-mediated basal recycling of PINs (Geldner et al., 2003; Kleine-Vehn et al., 2008a), to dissect the basal and apical recycling pathways in plants. Elevated levels of PID, WAG1 or WAG2 in their endogenous epidermal expression domain in the co-presence of BFA reduced normally observed PIN1 and PIN2 entrapment within BFA compartments, as evidenced by formation of much smaller and rapidly disappearing PIN-positive BFA compartments as compared with the BFA treatment alone (Fig. 6A-D,J,K). This is in line with the recently shown effect of ectopically expressed PID on PIN1 localization (Kleine-Vehn et al., 2009). In accordance with reduced localization of PIN to the BFA compartments in excess PID, PIN2 persistence into BFA compartments in root epidermis cells was significantly enhanced (as measured by the extent of PIN2 at the PM) in the *pid wag1 wag2* triple mutant as compared with WT or the *wag1 wag2* double mutant (Fig. 6E-G; see Fig. S5 in the supplementary material), and loss-of-phosphorylation PIN2V SA showed enhanced persistence in BFA compartments as compared with PIN2V (Fig. 6H,I). Root epidermis expressed PIN1-EosFP2 is basal as opposed to apical PIN2, probably owing to affinity differences of the AGC3 kinases for the respective PINHLs. PIN1-EosFP2 shifted its localization from basal-to-apical after prolonged BFA treatment (Fig. 6L). However, this BFA-induced PIN1-EosFP2 apicalization did not occur in the *pid wag1 wag2* mutant and, instead, PIN1-EosFP2 remained less polar (Fig. 6M). Also in *cpi1* mutant roots (with reduced PM localized PID), prolonged BFA treatment did not lead to a basal-to-apical shift, but rather to less-polar PIN1-EosFP2 localization (Fig. 6N,O). Together, these results confirm the redundant action of PID, WAG1 and WAG2, and indicate that PIN phosphorylation by these three kinases reduces their affinity for the basal, GNOM-dependent recycling pathway and instructs their recruitment in the GNOM-competitive apical recycling pathway.

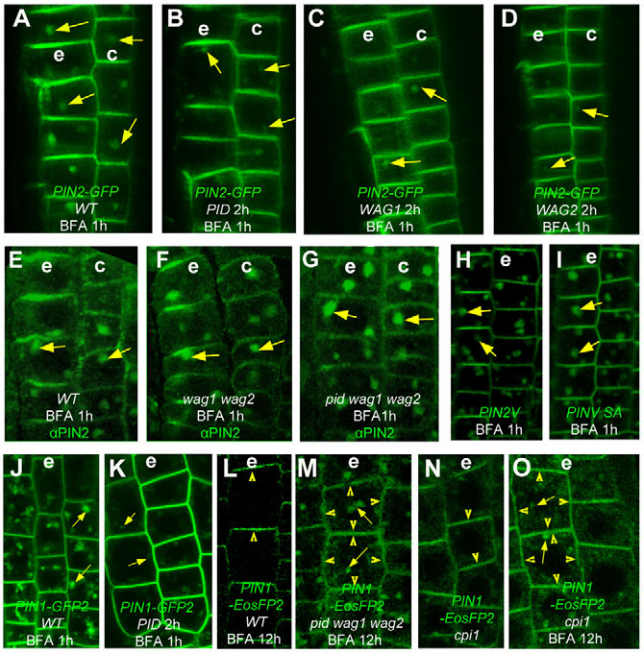


**Fig. 6. PID, WAG1 and WAG2 kinases recruit PINs to the apical recycling pathway by reducing their affinity for the BFA-sensitive basal recycling pathway.** (**A-D**) Two hours *G10-90::XVE>>PID* (B), *G10-90::XVE>>WAG1* (C) and *G10-90::XVE>>WAG2* (D) induction makes *PIN2::PIN2-GFP* less sensitive to basal recycling inhibitor BFA, as compared with the control (A). (**E-I**) PIN2 accumulation into BFA compartments in root epidermis cells is enhanced in the *pid wag1 wag2* triple mutant (G) as compared with WT (E) or the *wag1 wag2* double mutant (F). Also, loss-of-phosphorylation *PIN2::PIN2V SA* (I) displays enhanced accumulation in these compartments as compared with *PIN2::PIN2V* (H). (**J**,**K**) Induction of *G0-90::XVE>>PID* expression (K) recruits basal *PIN2::PIN1-GFP2* to the apical targeting pathway as evidenced by formation of smaller and rapidly disappearing PIN1-positive BFA compartments (before their eventual translocation to the apical side) as compared with the control (J). (**L**,**M**) *PIN2::PIN1-EosFP2* (green) apicalization by 12 hours BFA treatment (L) is impaired in *pid wag1 wag2* (M). (**N**,**O**) BFA-induced apicalization of basally localized *PIN2::PIN1-EosFP2* (N) is reduced in the sterol biosynthesis mutant *cpi1* (O). Yellow arrows depict BFA compartments and yellow arrowheads show PIN polarity.

## AGC3 kinases act after non-polar PIN secretion to the PM to promote apical PIN recycling

We used a *PIN2::PIN1-EosFP2* fusion to track the real-time relocation of basally localized PINs to the apical cell side in root epidermis cells. Photoconversion of PIN1-EosFP2 from green to red at the basal side of epidermis and simultaneous induction of PID directly demonstrated PID-induced PIN transcytosis (translocation of PM cargo from one polar domain to another; Fig. 7C). Interestingly, upon reversal of PID induction (by washing out the induction medium), apically localized PIN2::PIN1-EosFP2 returned to the basal PM domain (Fig. 7D), indicating that constitutive PID action is required for maintaining apical PIN localization.

Next, we assessed polarization of newly synthesized PINs in WT and kinase loss- and gain-of-function mutant backgrounds. Basal localization of PIN1 is established by non-polar secretion followed by basal recycling (Dhonukshe et al., 2008) (Fig. 7M,N). The secretion of induced PIN2 was also non-polar and was followed by its translocation to the apical cell side in WT (Fig. 7O,P), whereas it translocated to the basal side in the *pid wag1 wag2* triple mutant (Fig. 7Q,R). PIN1 secretion was initially symmetric in excess PID and, instead of going basal, PIN1 went from non-polar to the apical cell side (Fig. 7S,T). This corroborates that PID, WAG1 and WAG2 do not act during the initial PIN secretion but only when the PINs colocalize with the kinases at the PM, and indicates that, in the epidermis, efficient PIN1 phosphorylation by excess PID triggers its translocation from non-polar to the apical side similar to that of PIN2 with endogenous PID, WAG1 and WAG2 levels.

Finally, to visualize PID, WAG1 and WAG2 kinase action on oppositely localized PINs in the same cell, we analyzed lines coexpressing *PIN2::PIN1-GFP2* (basal in epidermis and cortex) (Wisniewska et al., 2006) and *PIN2::PIN2-mCherry* (apical in epidermis and basal in cortex) (Fig. 7A). Upon PID induction, only PIN1 in the epidermis and both PIN1 and PIN2 in the cortex showed apicalization (Fig. 7B), reinforcing that *PID* gain-of function maintains apically localized PINs, and shifts basally localized PINs to the apical domain.

## PID-, WAG1- and WAG2-triggered apical PIN transcytosis involves the endocytosis-associated Rab5 pathway

Coexpression of PID and the dominant-negative *Arabidopsis* Rab5 homolog (DN-Ara7), which previously has been shown to reduce PIN endocytosis in roots (Dhonukshe et al., 2008), did not affect

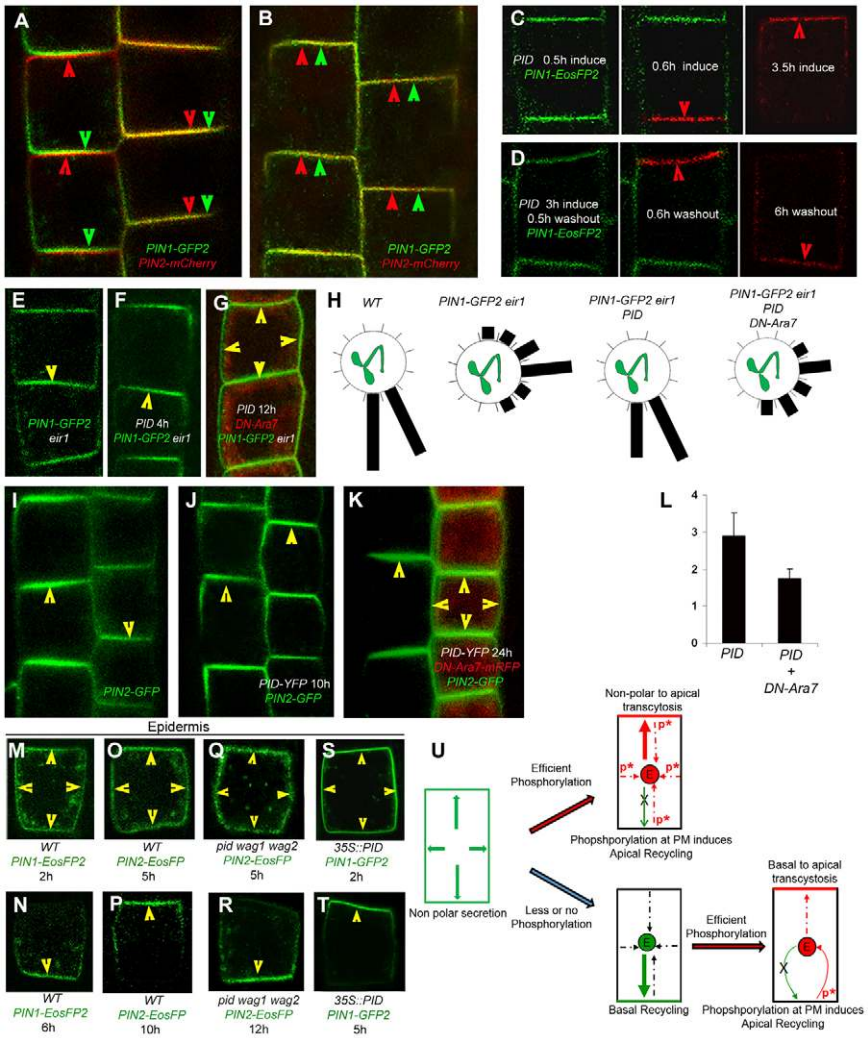


**Fig. 7. The endocytosis-associated Rab5 pathway is required for PID, WAG1 and WAG2 phosphorylation-triggered basal-to-apical and non-polar-to apical PIN transcytosis.** (**A**,**B**) *G10-90::XVE>>PID* induction specifically affects the basal cargo (*PIN2::PIN1GFP2* in epidermis and cortex and *PIN2::PIN2mCherry* in cortex) for its apical relocation. (**C**,**D**) Green-to-red photoconverted *PIN2::PIN1-EosFP2* shows basal-to-apical transcytosis upon *WER::XVE>>PID* induction by estradiol (C) and displays apical-to-basal relocation after estradiol washout (D). (**E-G**) *WER::XVE>>PID* is unable to induce basal-to-apical transcytosis of *PIN2::PIN1-GFP2* (green) in the presence of endocytosis-impairing *WER::XVE>>DN-Ara7-RFP* (red). (**H**) Root gravitropic response histogram showing that *WER::XVE>>PID* induction rescues the gravity defects of *pin2 PIN2::PIN1GFP2* by PIN1 apicalization in the epidermis, whereas co-induction of the endocytic inhibitor *WER::XVE>>DN-Ara7-RFP* annihilates this rescue. (**I-L**) Cortex-specific *CO2::XVE>>* driven expression of DN-Ara7-mRFP (red) inhibits PID-mediated apicalization of *PIN2::PIN2-GFP* (green, compare J with K). (L) PIN2 polarity index (ratio of apical to lateral PIN2-GFP intensity) in the cortex. Bars show means and s.d. (**M-T**) Induction of *WER::XVE>>PIN1-EosFP2* (N) or *WER::XVE>>PIN2-EosFP* (P) expression in the epidermis first results in non-polar secretion (M,O) followed by recruitment to the basal or the apical PM (N,P), respectively. Inducible expression of *WER::XVE>>PIN2-EosFP* in *pid wag1 wag2* results in non-polar (Q) to largely basal (R) PIN2 trafficking. Non-polarly secreted *WER::XVE>>PIN1-GFP2* (green, S) gradually relocates to the apical membrane in *35S::PID* (T). Time of induction of expression is indicated. Arrowheads show PIN polarity. (**U**) Model for PID-, WAG1- and WAG2-kinase action: non-phosphorylated PIN proteins (green) arrive at the PM in a non-polar manner; phosphorylation at the PM (red) triggers non-polar or basal-to-apical PIN trafficking.

the abundance and normal non-polar localization of PID (not shown) but led to symmetric PIN2-GFP localization in the cortex (Fig. 7I-L). Moreover, epidermis-specific coexpression of DN-Ara7 inhibited PID-triggered basal-to-apical transcytosis of PIN2::PIN1-GFP2, which is required for rescuing the agravitropic *pin2* phenotype (Wisniewska et al., 2006) (Fig. 7E,F,H; see Fig. S6A-F in the supplementary material). Instead, PIN1-GFP2 became apolar and was unable to rescue the agravitropic phenotype (Fig. 7G,H; see Fig. S5C,F in the supplementary material). These data show that PID-driven basal-to-apical PIN transcytosis requires the endocytosis-related Rab5 pathway.

## DISCUSSION

### *Arabidopsis* PID, WAG1 and WAG2 kinases are PIN polarity determinants involved in both programmed and environmentally regulated development

Previously, we have shown that the PID kinase and PP2A phosphatase act antagonistically on PIN polarization through reversible phosphorylation of the PINHL (Friml et al., 2004; Michniewicz et al., 2007). Here, we identified two PID-related kinases, WAG1 and WAG2, as novel PIN polarity regulators. In the embryo and the root, the WAG kinases act redundantly with PID,

with the same phosphorylation specificity and ability to regulate apical PIN localization. Our results are in line with the previously reported functional analysis of *WAG1* and *WAG2* (Cheng et al., 2008; Santner and Watson, 2006), but are seemingly contradictory to the observed inverse regulation of *PID* and *WAG2* expression during valve margin specification in fruits that correlated with PIN3 polarity changes (Sorefan et al., 2009). This suggests that the effect of the kinases on PIN polarity might depend on tissue-specific factors, which might be different in embryos or seedlings than in fruits.

PID and the WAG kinases belong to the plant-specific AGCVIII family of kinases, within which they cluster into the AGC3 subfamily (see Fig. S1 in the supplementary material). Testing kinases from other AGC subfamilies showed that PID, WAG1 and WAG2, but not these other AGC kinases, can induce a basal-to-apical shift in PIN polarity. Our results corroborate a previous functional analysis of the D6 kinases (AGC1-1, AGC1-2, PK6 and PK5), which seem to regulate polar auxin transport but do not affect PIN polarity (Zourelidou et al., 2009).

Based on the phenotypes of the *pid* loss-of-function mutant in the embryo and inflorescence, the PID kinase has initially been considered as regulator of programmed plant development (Benjamins et al., 2001; Christensen et al., 2000). We extend this notion by showing that PIN polarity is apical instead of basal in the outer layers of the root owing to tissue-specific expression of PID, WAG1 and WAG2. However, the strong wavy root phenotype of the *wag1 wag2* double mutant (Santner and Watson, 2006), the mild agravitropy of *pid* mutant roots (Sukumar et al., 2009) and our observation that *pid wag1 wag2* mutant roots are strongly affected in both wavy and gravitropic growth also point to an important role for these three kinases in adaptive plant development. The impairment of apical PIN2 polarity in the absence of PID, WAG1 and WAG2 leads to altered auxin distribution, resulting in a reduced root meristem size and agravitropic root growth.

## Kinase-instructed basal-to-apical transcytosis competes with GNOM-dependent basal recycling

Previously, we have shown a two-step mechanism for generation of basal PIN polarity in plants (Dhonukshe et al., 2008). Our current results now identify a two-step mechanism that generates apical PIN polarity in which non-polar PIN secretion is followed by PID-, WAG1- or WAG2-instructed apical PIN recycling. GNOM-based basal recycling acts predominantly in the generation of basal PIN localization, whereas PID-, WAG1- and WAG2-based apical recycling acts predominantly in the generation of apical PIN polarity. Therefore, we propose that the phosphorylation status-dependent relative contribution of both pathways determines whether PIN will traffic towards the apical or basal cell side.

In analogy, transcytosis of certain mammalian proteins also depends on phosphorylation (Casanova et al., 1990). Some of these transcytosed proteins, such as the polymeric immunoglobulin receptor, pass through retromer compartments (Verges et al., 2004). Our results, conversely, show that in plant cells PID and WAG kinase-instructed transcytosis and apical recycling of PIN proteins occurs in the absence of the VPS29- and SNX1-dependent retromer complex, which is in accordance with the finding that the basal-to-apical shift by prolonged BFA treatment occurs independent of the retromer complex (Kleine-Vehn et al., 2008a). This, together with the previous observation that GNOM-mediated basal recycling of PIN proteins also occurs independent of SNX1 (Kleine-Vehn et al., 2008b), indicates that plant PIN proteins do not pass through retromer compartments during their transcytosis or recycling. Instead, the plant retromer seems to be involved in recruiting PIN proteins from the prevacuolar compartments back to the recycling pathway (Kleine-Vehn et al., 2008b), which might explain the observed enhanced vacuolar accumulation of PIN proteins in *vps29* root cells (Jaillais et al., 2007).

PID, WAG1 and WAG2 are all expressed predominantly in the root epidermis; a cell layer that bears PIN2 on the apical cell side and PIN1 on the basal cell side. PIN2 localizes to the apical cell side immediately after its non-polar secretion with default PID, WAG1 and WAG2 amounts, whereas PIN1 does the same only when PID, WAG1 or WAG2 is overexpressed. Once the level of PID, WAG1 or WAG2 is increased, the basally localized PIN1 is recruited by the apical recycling pathway for its gradual basal-to-apical transcytosis. These data suggest that PIN2 might be a more favored substrate for these kinases than PIN1. This notion is further supported with the observation that PIN1 is localized to the apical side of the epidermal cells in a *pin2* mutant (Vieten et al., 2005).

## Basal-to-apical PIN transcytosis requires the endocytosis-related Rab5 pathway

By real-time tracking of PINs using green-to-red photoconvertible tag EosFP (Dhonukshe et al., 2007), we directly visualized PID-, WAG1- and WAG2-mediated PIN transcytosis. Impairment of the endocytosis-related Rab5 pathway interfered with PID-, WAG1- and WAG2-induced PIN transcytosis, indicating its involvement in kinase-mediated PIN polarity establishment. Therefore, we conclude that, as a general regulator, the Rab5 pathway not only operates in trafficking of PINs to the basal recycling pathway (Dhonukshe et al., 2008) but also functions in trafficking of phosphorylated PINs to the apical PM.

Previous detailed pulse-chase analysis of the endocytic tracker FM4-64 has determined the localization of Ara7 (a plant homolog of mammalian Rab5) on an early type of endosomes (Ueda et al., 2004), and more recent immunogold electron microscopy results have shown its localization to multivescicular body compartments (Haas et al., 2007). Therefore, the mechanism by which Rab5 pathway impairment leads to reduced endocytosis is unclear. Despite this, the DN-ARA7 provides one of the very few available genetic tools to impair PIN endocytosis, especially when PIN proteins pass through the Ara7-positive endomembrane compartments (P.D. and B.S., unpublished) (Furutani et al., 2007). It will be an important future task to resolve the step-wise trafficking of PINs along the plant endomembrane compartments and the phosphorylation status-based differential sorting stations.

## Mechanism of PID and WAG kinase action

Our results implicate a role for PM composition in effective PIN polarity regulation by PID, WAG1 and WAG2. In the sterol mutant *cpi1* the localization of PID at the PM was reduced, and PID-triggered PIN apicalization was not observed within a 20-hour timeframe. These observations concur with a previous report showing that PIN2 apicalization in the epidermis is slowed down in the *cpi1* mutant (Men et al., 2008). This further matches with our finding of the predominant expression of PID, WAG1 and WAG2 within the root epidermis. Accordingly, the *cpi1* mutant shows the gravity defects (Men et al., 2008). In view of the pleiotropic defects of the *cpi* mutant such as strong dwarf phenotypes, we are aware that this might be indirect evidence.

The *cpi1* mutant data, together with our tracking results and colocalization analysis, suggest that PID, WAG1 and WAG2 act on PINs predominantly at the PM. It has been shown before that the prolonged treatment with the GNOM inhibitor BFA induces a

basal-to-apical switch of PIN1 (Kleine-Vehn et al., 2008a) and that, in the *pid* mutant, the BFA-induced PIN apicalization is slower (Kleine-Vehn et al., 2009). As prolonged treatment of BFA is unable to induce basal-to-apical PIN relocation in *pid wag1 wag2*, our results now show the importance of PID, WAG1 and WAG2 in that process, which confirms the redundant action of the three kinases.

Our previous results have shown the mechanism for generation of basal PIN localization (Dhonukshe et al., 2008). The data described here and in the manuscript by Kleine-Vehn and coworkers (Kleine-Vehn et al., 2009) now identify the decisive steps that lead to apical PIN polarity. Newly synthesized PINs arrive at the PM in a non-polar manner, where, upon less efficient or no phosphorylation, they are recruited to the GNOM-dependent basal recycling pathway, leading to basal PIN polarity. Phosphorylation of non-polar or basally localized PINs by the non-polarly PM localized PID or WAG kinases at specific residues enhances PIN affinity for the GNOM-competitive apical recycling pathway. Thus, apical PIN localization is achieved either by non-polar-to-apical or basal-to-apical PIN translocation (Fig. 7U). This now provides further insight into how the apical and basal PIN trafficking pathways in plants are regulated and identifies the WAG kinases as new key regulators that instruct PIN polarity-based auxin fluxes to modify plant development.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

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