CORRECTION

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

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The authors informed us of problems related to Fig. 3C and Fig. 7D in Development 137, 3245-3255. Both issues were noted by an investigation by the Technical Committee of Utrecht University (UTC) into this publication. Based on the findings provided (below), the journal has decided that the major conclusions of the paper are not affected and that retraction is not required, but that a correction should be provided with an explanation of the circumstances. This course of action complies with our policy on correction of issues in the scientific record, which states: “Should an error appear in a published article that affects scientific meaning or author credibility but does not affect the overall results and conclusions of the paper, our policy is to publish a Correction”.

For Fig. 3C, the UTC concluded that it was the result of “cut-and-paste”, which is “not indicated by a solid line, nor is it mentioned in the figure legend, as prescribed by journal policy”. Fig. 3C presents results of in vitro phosphorylation of the different PIN2 hydrophilic loop (PIN2 HL) versions by the kinases PID, WAG1 and WAG2. The results were more or less similar for the three kinases (Fig. 3 revised version), and in order to reduce the figure size, it was decided that it would be sufficient to show the full data for the PINOID kinase, and to focus on the phosphorylation results for the wild-type version and the loss-of-phosphorylation version of the PIN2 HL for the WAG1 and WAG2 kinases. This involved splicing, and although the splicing is clearly visible in the original Fig. 3C, the appropriate presentation according to the standards of the journal would have been to leave space between the spliced parts. As demonstrated by the comparison between the revised Fig. 3 (below) and the original Fig. 3C, the spliced version represents part of the original data. In the revised version of Fig. 3 the full data set is shown and a more detailed description of the results is provided in the revised figure legend text.

With respect to Fig. 7D, the UTC noted that “it is not possible that random background noises, taken from two images, are identical. The most likely explanation is that the same green panel was used for the left and middle panel of Fig. 7D”. The original data were not available to the UTC, and this anomaly could not be resolved. Development also appointed its own independent expert to analyse the images, who concluded that “the two images are almost identical (except for the region purporting to show photoconversion) and it is highly unlikely that these could represent different time points. The analysis suggests that one of these images appears to have been generated by manipulating the other.” For this reason the authors and editors have seriously considered retraction of Development 137, 3245-3255. However, the thorough analysis by the UTC and the Development editors identified no other abnormalities in the data. The authors and editors therefore feel that removing Fig. 7D and the related text from the publication provides the optimal solution for protecting the integrity of the scientific record.

According to the authors: “The main conclusion of the paper is that the AGC3 kinases PID, WAG1 and WAG2, and not other plant AGC kinases, instruct recruitment of PINs into the GNOM-independent apical recycling pathway by phosphorylating the middle serine in three conserved TPRXS(N/S) motifs. Based on data provided in Development 137, 3245-3255 and also in other publications (Friml et al., 2004, Science 306, 862-865; Michniewicz et al., 2007, Cell 130, 1044-1056; Kleine-Vehn et al., 2008, Curr. Biol. 18, 1-6; Kleine-Vehn et al., 2009, Plant Cell 21, 3839-3849; Huang et al., 2010, Plant Cell 22, 1129-1142), a model is proposed by which apolarly localized kinases phosphorylate PINs at the plasma membrane after PIN secretion, and trigger endocytosis-dependent PIN transcytosis (the trafficking of existing PIN cargo from one polar domain to another) to the apical domain. The objective of the photoconversion and time-lapse imaging experiments presented in Fig. 7D was merely to provide evidence for phosphorylation-triggered PIN transcytosis. PIN transcytosis was reported before by Kleine-Vehn et al. in 2008, Curr. Biol. 18, 1-6; and in 2009, Plant Cell 21, 3839-3849, and a role for PID in this process was also proposed, but not definitively demonstrated, in that latter paper.” Omission of Fig. 7D does not affect the main conclusions, and given that phosphorylation-triggered PIN transcytosis is only one minor aspect of the full paper, the editorial board of Development has
decided that a correction suffices. The authors agree with this decision. The readers of Development 137, 3245-3255 are therefore advised to ignore the data presented in Fig. 7D and the related text:

P. 3251: “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.”

P. 3252, legend Fig. 7: “Green-to-red photoconverted PIN2::PIN1-EosFP2 displays apical-to-basal relocation after estradiol washout (D).”

Pankaj Dhonukshe, the co-first and co-corresponding author of the original manuscript, is not listed as an author on this Correction upon his request. He provided us with the following statement, expressing his opinion:

“Pankaj Dhonukshe does not agree with the content of the Correction Notice as:

i. the anomaly in the first green panel (before photoconversion) at Fig. 7D does not affect the published conclusion drawn from research findings. The conclusion is solely based and derived from separately acquired red panels shown in Fig. 7D. Deriving conclusions from only red panels is a standard practice.

ii. The anomaly at Fig. 7D does not affect the published conclusion. The current content related to Fig. 7D should therefore be revised, and not removed.

iii. For Fig. 3C, original data did not accurately support the published conclusion. As per the Correction Notice, original data not previously shown is included and new conclusions are derived from this data. Readers should be aware that original conclusions are therefore not accurately supported.”

We note that the affiliations of several authors have been updated in the author list above.
Plasma membrane-bound AGC3 kinases phosphorylate PIN auxin carriers at TPRXS(N/S) motifs to direct apical PIN recycling

Pankaj Dhonukshe1,*,§, Fang Huang2,*, Carlos S. Galvan-Ampudia2,*, Ari Pekka Mähönen1,†, Jurgen Kleine-Vehn3, Jian Xu1,‡, Ab Quint2, Kalika Prasad1, Jiří Friml3, Ben Scheres1 and Remko Offringa2,§

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 PIN 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 hydrophilic 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 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_020505), wag2 (SALK_070240) (Cheng et al., 2008; Santner and Watson, 2006), ein1-1 (Luschning et al., 1998), 35S::PID (Benjamins et al., 2001), PID::PID-Venus (Michniewicz et al., 2007), gnom (Geldner et al., 2004), snxl (Jailaiss et al., 2006), yps29 (Jailaiss 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 (Asab et al., 2006) and all T-DNA constructs (see below) were introduced into Agrobacterium tumefaciens strain C58C1 (GV3101) (Van et al., 1974) or Agrobacterium strain C58C1 (GV3101) (Van et al., 2006) and all T-DNA constructs (see below) were introduced into Arabidopsis AGL1 (Lazo et al., 1991) by electroporation. Plant transformation was performed by Agrobacterium tumefaciens strain C58C1 (GV3101) (Van et al., 1974) or Agrobacterium strain C58C1 (GV3101) (Van et al., 2006) and all T-DNA constructs (see below) were introduced into Arabidopsis AGL1 (Lazo et al., 1991) by electroporation. Plant transformation was performed as described (Dhonukse et al., 2007). Gene-specific primers are listed in Table S1 in the supplementary material.

Molecular cloning

Primers used for cloning are listed in Table S1 in the supplementary material. The coding region of WAG1 and WAG2 comprising, respectively, 3205 bp and 3402 bp upstream from the ATG, was amplified from Arabidopsis thaliana Col-0 genomic DNA. The cloning has been described previously (Zuo et al., 2000). Promoters, coding regions and terminators were cloned into binary vectors (Hellens et al., 2000) for constitutive overexpression, the Gateway recombination cassette was kindly provided by Christian Roth (BOKU, Vienna, Austria). For the site-directed mutagenesis, we employed the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene). The oligonucleotides used to introduce mutations in the PIN2HL cDNA and the PIN2HL promoter were excised from pPIN2F and cloned into vector pBlueScript. GFP was replaced with EosFP to have PIN1-EosFP2. PIN1-EosFP2 was PCR amplified with 221PINF1 and 221PINR1 to bring it into second box of Multisite Gateway. PIN1-EosFP2 cloning has been previously described (Dhonukse et al., 2007). PIN1-EosFP2 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-DNAr7 was generated from GFP-DNAr7 (Dhonukse et al., 2006) by replacing the GFP with the mRFP coding region. mRFP-DNAr7 was PCR-amplified with primers 221mRFPF and 221mRFP2 to bring into the second box of Multisite Gateway and thereafter the gene cassette 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 MTB buffer as described previously (Friml et al., 2003) using an InSituPro robot (INTA VIS, Cologne, Germany). Rabbit anti-PIN1 (Friml et al., 2004), anti-PIN2 (Asab 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 (Dhonukse et al., 2006; Dhonukse 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 AGC-VIII 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 (Benjamin 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 triple mutant, 99% did not produce a cotyledon (Fig. 1G). In each double mutant, the number of missing cotyledons was consistent with the number of mutant alleles present (Fig. 1G). These results indicate that the three AGC3 kinases act redundantly on PIN1 and that their activity is crucial for proper cotyledon development.

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 waving (Fig. 1D-F) and suggested defects in PIN1 polarization. Immunolocalization showed that PIN1 polarity was predominantly basal with some lateral localization in epidermal cells of triple mutant embryos (Fig. 1G,H), whereas it was apical in wag1 wag2 embryos (Fig. 1I,J), 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 summary, PID and WAG kinases redundantly instruct apical PIN polarity during cotyledon and root development.
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 AGC 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 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.
To investigate the biological significance of the AGC3 kinase-dependent phosphorylation of 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 mutant form, is phosphorylated by GST-WAG1 or GST-WAG2.

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. 4ML). 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. 4PN), demonstrating that the loss-of-phosphorylation protein was insensitive to kinase overexpression. In the stelar 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(S/N) 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 steroid-induced kinase expression as compared with the WT (Fig. 5L).

**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., 2007) 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.

**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::PIN1-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
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 (Benjamin 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 wavey root phenotype of the wag1 wag2 double mutant (Santner and Watson, 2006), the mild agrivitropy 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 agrivitropic 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 (Jailais 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 increased. 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 multivesicular 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.

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 (Dhonukse 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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ACG3 kinases direct apical PIN polarity

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