The polarly localized D6 PROTEIN KINASE is required for efficient auxin transport in \textit{Arabidopsis thaliana}

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The phytohormone auxin is a major determinant of plant growth and differentiation. Directional auxin transport and auxin responses are required for proper embryogenesis, organ formation, vascular development, and tropisms. Members of several protein families, including the PIN auxin efflux facilitators, have been implicated in auxin transport; however, the regulation of auxin transport by signaling proteins remains largely unexplored. We have studied a family of four highly homologous AGC protein kinases, which we designated the D6 protein kinases (D6PKs). We found that \textit{d6pk} mutants have defects in lateral root initiation, root gravitropism, and shoot differentiation in axillary shoots, and that these phenotypes correlate with a reduction in auxin transport. Interestingly, D6PK localizes to the basal (lower) membrane of \textit{Arabidopsis} root cells, where it colocalizes with PIN1, PIN2 and PIN4. \textit{D6PK and PIN1} interact genetically, and D6PK phosphorylates PIN proteins in vitro and in vivo. Taken together, our data show that D6PK is required for efficient auxin transport and suggest that PIN proteins are D6PK phosphorylation targets.

**KEY WORDS:** \textit{Arabidopsis}, Protein kinase, Auxin transport, PIN proteins, Lateral root

\textbf{INTRODUCTION}

Plant development is to a large extent dependent on the proper distribution of the hormone auxin (indole-3-acetic acid, IAA). IAA can enter plant cells via membrane diffusion in its protonated form (IAAH) or via active transport in its anionic form (IAA$^-$); IAA, the predominant form of IAA in cells, is exported via auxin efflux carriers (Kramer and Bennett, 2006). The permease-like AUXIN-RESISTANT 1 and LIKE-AUX1 (AUX1 and LAX) proteins are auxin influx (Bridge et al., 2008; Marchant et al., 1999; Yang et al., 2006) and the PIN-FORMED (PIN) proteins are auxin efflux facilitators (Kramer and Bennett, 2006; Petrasek et al., 2006; Teale et al., 2006; Vieten et al., 2007). AUX1 and PIN proteins have been shown to promote auxin influx and efflux, respectively (Chen et al., 1998; Luschnig et al., 1998; Petrasek et al., 2006; Yang et al., 2006). In addition, it has been proposed that PIN proteins in planta act together with MULTIDRUG RESISTANCE/PHOSPHOGLYCOPROTEIN (MDR/Pgp) ATP-binding cassette transporters (Bandyopadhyay et al., 2007; Blakeslee et al., 2007; Geisler and Murphy, 2006).

The directionality of auxin transport within the plant is achieved by the differential and often polar localization of the AUX1/LAX and PIN transport facilitators (Benkova et al., 2003; Friml et al., 2002a; Friml et al., 2003; Friml et al., 2002b; Galweiler et al., 1998; Kleine-Vehn et al., 2006; Muller et al., 1998; Sauer et al., 2006; Swarup et al., 2001; Wisniewska et al., 2006). Mutations in \textit{AUX1}/\textit{LAX} genes have been reported to affect gravitropism, lateral root formation, and phyllotaxy (Bridge et al., 2008; Bennett et al., 1996; Marchant et al., 2002; Marchant et al., 1999; Swarup et al., 2008). Mutations in single \textit{PIN} genes affect shoot differentiation, vascular development, lateral root development, and tropisms (Benkova et al., 2003; Chen et al., 1998; Friml et al., 2002b; Galweiler et al., 1998; Luschnig et al., 1998; Muller et al., 1998; Okada et al., 1991; Scarpella et al., 2006), whereas mutants defective in multiple \textit{PIN} genes have more pronounced phenotypes and affected embryonic development, root patterning, and lateral root initiation (Benkova et al., 2003; Bilou et al., 2005; Friml et al., 2003). PIN proteins have redundant functions, and the loss of one PIN protein is compensated for by the ectopic activities of the other PIN family members (Bilou et al., 2005; Paponov et al., 2005; Vieten et al., 2005). Interestingly, \textit{pin} mutant phenotypes can in many cases be mimicked by the application of the auxin efflux inhibitor naphthylphthalamic acid (NPA) (Katekar and Geissler, 1977), indicating that this inhibitor functions in the proximity of PINs.

PIN polarity is controlled by differential PIN phosphorylation, which appears to be the result of the antagonistic activities of the serine-threonine kinase PINOID (PID) and a phosphatase containing the subunit PP2A (Friml et al., 2003; Michniewicz et al., 2007). \textit{PID} overexpression leads to a shift in the polarity of at least \textit{PIN1}, \textit{PIN2} and \textit{PIN4} from the basal (lower) to the apical (upper) membrane in root cortex and lateral root cap cells (Friml et al., 2003). These \textit{PID}-dependent polarity changes can be reverted by increasing the expression of \textit{PP2A}, which suggests that PIN polarity and auxin efflux are at least in part controlled by \textit{PID}-dependent phosphorylation (Michniewicz et al., 2007).

The plant-specific AGC kinases were named on the basis of their homology to the mammalian cAMP-dependent protein kinase A, cGMP-dependent protein kinase G and phospholipid-dependent protein kinase C (Bogre et al., 2003). PID together with 22 other protein kinases forms the AGCVIII subgroup of the \textit{Arabidopsis} AGC kinase family (Bogre et al., 2003; Galvan-Ampudia and Offringa, 2007). AGCVIII kinases are characterized by a DFG to DFD substitution in the conserved catalytic subdomain VII, as well as by the presence of a conserved insertion between subdomains VII and VIII of the kinase. In addition to PID, other protein kinases of the AGCVIII subgroup have been examined, including the blue light
receptors PHOTOTROPIN 1 (PHOT1) and PHOT2, and the root growth regulators WAVY ROOT GROWTH 1 (WAG1) and WAG2 (Sakai et al., 2001; Santner and Watson, 2006). Although the precise molecular mechanisms that control PHOT-mediated phototropism and WAG-mediated root waving remain to be elucidated, changes in auxin transport or auxin response may well be responsible for these growth responses (Esmon et al., 2006; Harper et al., 2000; Santner and Watson, 2006).

MATERIALS AND METHODS

Biological material
d6pk mutant lines (Arabidopsis thaliana Columbia) were identified in the SIGNAL database (http://signal.salk.edu/cgi-bin/sallexpress) and obtained from the Nottingham Arabidopsis Stock Centre. d6pk-1 (SALK_061847), d6pk-2 (SAIL_242_C05), d6pkkl1-1 (SALK_056618), d6pkkl2-1 (SALK_005798), d6pkkl2-2 (SALK_086127), d6pkkl3-1 (SALK_011507), d6pkkl3-2 (SALK_047347) and d6pkkl3-3 (SALK_081961). The pin1 (SALK_097144) allele was identified and obtained in the same manner. The alleles d6pk-1, d6pkkl1-1, d6pkkl2-2 and d6pkkl3-2 were chosen for further biological and genetic analyses. The sequences of the primers used for genotyping can be provided on request. DR5:GUS transgenic lines were a gift from Tom Guilfoyle (University of Columbia, MO, USA) (Ulmasov et al., 1997); the PIN1:GFP construct was obtained from Jiri Friml (Gent, Belgium) (Benkova et al., 2003). To introduce the pin1 mutation and the DR5:GUS reporter into the d6pk and YFP:D6PK backgrounds, pin1/PIN1 plants or DR5:GUS transgenic lines were crossed with d6pk/d6pk d6pkkl1/d6pkkl1/d6pkkl2/D6PKL2 and YFP:D6PK lines (DR5:GUS only). Relevant homozygous backgrounds were selected from the progeny of these crosses by phenotyping and genotyping.

Cloning procedures
To generate YFP:D6PK, the D6PK open reading frame was amplified by PCR with the primers D6PK-FW-GW1 and D6PK-RV-GW1 and inserted into the Gateway-compatible vector pEXTAG-YFP-GW (a gift from Jane Parker, Cologne, Germany). 35S:D6PK and D6PK-GFP were generated using Gateway-technology by insertion of D6PK fragments obtained with the primers D6PK-FW-GW1 and D6PK-RV-GW1 or D6PK-RV-GW2 into p355GW-MYC and MDC38 (Curtis and Grossniklaus, 2003). YFP:D6PK, D6PK-GFP and 35S:D6PK were transformed into Arabidopsis thaliana (Columbia) to obtain transgenic plants expressing the respective D6PK variants. YFP:D6PK and D6PK-GFP transgenic plants have identical morphology and the subcellular localization of D6PK:GFP is identical to the variants. YFP:D6PK and D6PK:GFP transgenic lines have identical p355SGW-MYC and pMDC83 (Curtis and Grossniklaus, 2003). YFP:D6PK, the primers D6PK-FW-GW1 and D6PK-RV-GW1 or D6PK-RV-GW2 into D6PK, to generate GST:PIN1. GST:D6PK was obtained by insertion of the bacterial expression of His-tagged PINLOOP fusion proteins, namely pDEST17 (Invitrogen, Carlsbad, CA, USA), to generate constructs for the system compatible fragments were then cloned into the expression vectors.

Physiological experiments
Unless otherwise stated, seedlings were grown in continuous light on standard growth medium [4.2 g/l Murashige and Skoog salts, 1% sucrose, 0.5 g/l 2-(N-morpholino)ethanesulfonic acid, 5.5 g/l agar, pH 5.8]. Older plants were grown in the greenhouse with 16-hour light/8-hour dark cycles. Sensitivity to 2,4D was measured in 11-day-old seedlings that had been transferred after 4 days to medium containing 0.1 μM 2,4D. Auxin-induced lateral root formation was examined in seedlings that had been transferred after growth for 7 days on standard growth medium to medium supplemented with 0.05 μM NAA or 0.05 μM 2,4D. For microscopic analyses, plants were mounted on microscope slides with chloral hydrate:ddH2O:glycerol (20:9:3) and examined using an Axioshot microscope using Nomarski optics (Zeiss, Oberkochen, Germany).

Auxin transport
Following a previously published protocol, 25-mm inflorescence pieces were cut above the rosette of 3- to 4-week-old wild-type and d6pk d6pkkl2 mutants and placed in inverted orientation for one hour in 30 μl auxin transport buffer [500 μM IAA, 1% sucrose, 5 mM 2-(N-morpholino)ethanesulfonic acid, pH 5.5] with or without 100 μM NPA. The inflorescence pieces were subsequently transferred for 5 minutes to auxin transport buffer containing 11 kBq (417 nm) radiolabeled [3H]IAA (GE Healthcare, UK) and then placed into a tube containing only auxin transport buffer. After 2 hours, 5-mm segments were dissected from the inflorescence stem, the lowermost segment was discarded, and the remaining segments were macerated overnight in 300 μl Hydroxide of Hyamine 10-X (Packard Instrument Company, Meriden, CT, USA). The solution was neutralized by adding 300 μl acetic acid and the uptake of [3H]IAA was quantified using a Wallac WinSpectral 1414 Liquid Scintillation Counter (Perkin Elmer Life Sciences Waltham, MA, USA). Three replicate measurements were made for each genotype. The experiment was repeated three times with reproducible results and the result of one experiment is shown.

Gene expression
RT-PCR-based gene expression analysis was performed from RNA extracted from 7-day-old light-grown Arabidopsis seedlings with the RNeasy Kit (Qiagen). Total RNA (3 μg) was reverse transcribed with an oligo-dT primer using M-MuLV Reverse Transcriptase (Fermentas, St Leon, Germany). The consequences of the T-DNA insertions on the expression of the D6PK full-length transcripts were tested after 28 PCR amplification cycles. Auxin-induced gene expression of IAA genes was examined by RT-PCR using the same protocol. Primer sequences can be provided on request. D6PKp:GUS and DR5:GUS activity was detected by histological GUS staining of 7-day-old seedlings. For GUS staining, the seedlings were fixed for 15 minutes in heptane and stained for 30 minutes (DR5:GUS) or for 4 hours (D6PPGp:GUS) with GUS-staining solution [100 mM Na-phosphate buffer pH 7.0, 0.5 mM K3Fe(CN)6, 0.5 mM K4Fe(CN)6, 0.1% (v/v) Triton X-100, 0.5 mg/ml 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid], and subsequently destained in 70% (v/v) ethanol. For microscopic analyses, stained plants were mounted with chloral hydrate:ddH2O:glycerol (20:9:3) and examined using an Axioshot (Zeiss, Oberkochen, Germany) or a Leica MZ16 microscope (Leica Microsystems, Heerbrugg, Switzerland).

IAA measurements
Extraction and purification of IAA were carried out as previously reported (Edlund et al., 1995) with slight modifications as follows. Liquid nitrogen-frozen 7-day-old light-grown Arabidopsis seedlings were macerated overnight in 300 μl Hydroxide of Hyamine 10X, then filtered through filter paper, and the combined CH2Cl2 fraction was passed through a 0.2 μm filter. After centrifugation, the sample was resuspended in 20 μl H2O or H2O and 10 μl were injected into a liquid chromatography–electrospray ionisation-mass spectrometry system.
Spearman’s correlation coefficients were calculated using the ImageJ plugin confocal microscope (Leica Microsystems, Heerbrugg, Switzerland).

In vitro phosphorylation experiments were carried out using purified recombinant GST:D6PK and GST:D6PKin in combination with purified recombinant HIS:PIN proteins. The recombinant proteins were incubated for 30 minutes in phosphorylation buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM MgCl2, 0.01% Triton X-100, 1 mM DTT, 50 μM Ci [g-32P]ATP. The reaction was stopped by adding 5×Laemmli buffer, and protein extracts were separated on a 10% SDS-polyacrylamide gel (SDS-PAGE). GST:D6PK autophosphorylation and HIS:PIN transphosphorylation were detected by autoradiography, protein loading was controlled by immunoblots with anti-GST (GE Healthcare, UK) and anti-HIS (Sigma-Aldrich, Taufkirchen, Germany) antibodies.

RESULTS
d6pk mutant analysis suggests a role for D6PKs in auxin response or transport

We have examined the biological function of an AGCVIII subfamily, which comprises a protein that we designated D6 PROTEIN KINASE (D6PK; At5g55910, alternative nomenclature AGC1-1/PK64) and its close homologs D6PK-LIKE1 (D6PKL1; At4g26610, AGC1-2), D6PK-LIKE2 (D6PKL2; At5g47750, PK5) and D6PK-LIKE3 (D6PKL3; At3g27580, PK7; see Fig. S1 in the supplementary material) (Bogre et al., 2003; Galvan-Ampudia and O’farrill, 2007). The high degree of sequence conservation between these proteins strongly suggests that they have redundant function, and throughout this report we will refer to these four kinases collectively as D6PKs (see Fig. S1 in the supplementary material). By coincidence, D6PK is the closest Arabidopsis homolog of Phaseolus vulgaris (bean) PROTEIN KINASE-1 (PKP-1), the first protein kinase to be isolated from plants almost 20 years ago (Lawton et al., 1989).

To gain an understanding of the biological function of the D6PKs, we isolated and characterized homozygous T-DNA insertion mutants for each of the four D6PK genes (Fig. 1A). Because the alleles d6pk-1, d6pkl1-2 and d6pkl2-2 carry in-gene in-exon insertions and do not express detectable levels of the respective full-length transcript, we assume that these alleles are loss-of-function mutants (Fig. 1B). Based on the reduction of D6PKL1 expression in d6pkl1-1, the only available D6PKL1 allele, and based on the genetic interaction of d6pkl1-1 with other d6pk alleles (see below), we further concluded that d6pkl1-1 is a mutant with reduced D6PKL1 function (Fig. 1B). None of the d6pk single mutants displayed an obvious phenotype (Fig. 1C-G), but because the high degree of sequence conservation between the D6PKs suggested that these proteins have redundant biochemical function (see Fig. S1 in the supplementary material), and because their, at least in part, overlapping expression patterns indicated that they act in the same tissues (see Fig. S2 in the supplementary material), we decided to generate d6pk mutant combinations (Fig. 1H-M). When we analyzed these mutants, we observed a range of developmental defects, particularly in d6pk d6pkl1 d6pkl2, as well as in d6pk d6pkl1 d6pkl2 d6pkl3 mutants, which were absent in the single mutants and less pronounced in the d6pk double or the d6pk d6pkl1 d6pkl2 d6pkl3 triple mutants (Fig. 1H-M). Adult d6pk d6pkl1 d6pkl2 and d6pk d6pkl1 d6pkl2 d6pkl3 mutants had narrow and twisted leaves, formed fewer axillary shoots and were almost infertile (Fig. 1L-M); at the seedling stage, d6pk d6pkl1 d6pkl2 and d6pk d6pkl1 d6pkl2 d6pkl3 mutants sometimes had fused or single cotyledons (10% penetrance), were deficient in lateral root formation, and were mildly agraffitropic (Fig. 2). Our observation that mutants with reduced D6PK gene dosage display novel and increasingly stronger phenotypes supports our hypothesis that the four D6PK genes have redundant functions. To reduce the complexity of the subsequent experiments, further mutant analyses were largely restricted to the d6pk d6pkl1 d6pkl2 mutant.

Fused or single cotyledons, agraffitropic root growth, and reduced lateral root formation are phenotypes that are frequently observed in auxin response or transport mutants (Chen et al., 1998; Dr damarsi et al., 2005; Friml et al., 2002b; Fukaki et al., 2002; Luschnig et al., 1998; Muller et al., 1998; Scarpella et al., 2006). Auxin distribution can be estimated with the DR5::GUS reporter, which marks the sites of auxin (response) maxima, e.g. in lateral root founder cells and the root tip (Fig. 3A,E) (Benkova et al., 2003; Dubrovsky et al., 2008; Sabatini et al., 1999). In d6pk d6pkl1 d6pkl2 mutants, we found that DR5::GUS maxima are absent at the predicted sites of lateral root formation, and at the same time that the maximum in the root tip is broadened and shifted above the quiescent center (Fig. 3B,F). Because these phenotypes can, at least to some extent, be phenocopied by NPA-treatment of wild-type seedlings (Casimiro et al., 2001; Sabatini et al., 1999) (Fig. 3D,H), and because changes in the DR5::GUS maximum have also been reported for pin mutants (Friml et al., 2002a; Sabatini et al., 1999), we hypothesized that d6pk mutants might be deficient in auxin response or auxin transport.

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Immunostaining

Immunostaining was performed on roots of 5-day-old seedlings as previously described (Lauber et al., 1997), using mouse anti-GFP (dilution 1:300; Roche Applied Science, Indianapolis, IN, USA) for the detection of YFP:D6PK, rabbit anti-PIN1 [1:1000 (Paciorek et al., 2005)], rabbit anti-PIN2 [1:1000 (Abas et al., 2006)], and rabbit anti-PIN4 [1:200 (Friml et al., 2002a)] and secondary antibodies (anti-mouse FITC conjugate, dilution 1:600; and anti-rabbit Cy3-conjugate, dilution 1:600; both Dianova, Hamburg, Germany). DAPI (1 μg/ml) was used to stain nuclear DNA. For live imaging, propidium iodide (100 μg/ml) was used to outline cell walls. The effect of Brefeldin A (BFA; 50 μM) was analyzed following a 90-minute treatment as described previously (Geldner et al., 2003). All images were taken with a Leica TCS SP2 confocal microscope (Leica Microsystems, Heerbrugg, Switzerland). Spearman’s correlation coefficients were calculated using the ImageJ plugin from http://www.cibi.ac.uk/~afrench/coloc.html, according to French et al. (2008).
Reduced auxin transport in d6pk mutants

To distinguish between defects in auxin response and auxin transport in d6pk mutants, we conducted a number of physiological experiments. Each of our auxin response experiments led us to conclude that auxin responses are not compromised in d6pk d6pkl1 d6pkl2 mutants: auxin-induced gene expression was not affected in the mutants (see Fig. S3A in the supplementary material); mutant seedlings were indistinguishable from the wild type with regard to the auxin-induced inhibition of primary root growth (see Fig. S3B in the supplementary material); and, finally, lateral root formation along...
the primary root was efficiently induced in the mutants by the auxins 1-naphthalene acetic acid (1-NAA) and 2,4-dichlorophenoxyacetic acid (2,4D; see Fig. S3C in the supplementary material).

Interestingly, we observed a difference between d6pk d6pk1 d6pk2 mutants and the wild type with regard to lateral root initiation following NAA treatment. Although lateral root primordia were formed at regular intervals in untreated and in NAA-treated wild-type seedlings, lateral root primordia were often initiated close to a pre-existing primordium in d6pk d6pk1 d6pk2 mutants in response to NAA (see Fig. S3C in the supplementary material). NAA is known to enter cells by passive diffusion and therefore does not require auxin influx carrier activity (Delbarre et al., 1996). When we tested the sensitivity of d6pk mutants to the auxin transport inhibitor NPA, we found that d6pk d6pk1 d6pk2 mutant seedlings are hypersensitive to NPA, a result that might indicate that the mutants are auxin transport deficient (Fig. 3M). When we measured auxin transport in wild-type and in d6pk d6pk1 d6pk2 mutant stem segments using radiolabeled IAA, we revealed a strong decrease in IAA transport in the d6pk d6pk1 d6pk2 mutants (Fig. 3N). Because at the same time we did not detect any significant difference in the auxin content between the wild-type (15.2±1.3 ng IAA/g fresh weight; n=7) and d6pk d6pk1 d6pk2 mutant (14.3±3.1 ng IAA/g fresh weight; n=6) seedlings, we concluded that the d6pk d6pk1 d6pk2 mutant phenotype might be caused by an auxin transport defect.

**D6PK overexpression phenotypes**

To examine the effects of ectopic D6PK expression, we generated constructs for the overexpression of untagged D6PK (35S:D6PK) and of fluorescent protein-tagged D6PK (YFP:D6PK and D6PK:GFP). Interestingly, we failed to recover any 35S:D6PK transgenic plants, and we obtained only a small number of YFP:D6PK and D6PK:GFP overexpression lines after repeated rounds of transformation. Although our further experiments did not reveal any obvious differences in the biochemical activity of these D6PK variants (see below), we speculate that the overexpression of untagged D6PK causes lethality. YFP:D6PK and D6PK:GFP transgenic plants display identical phenotypes in that adult plants are shorter than the wild type, and in that they have shorter as well as broader, in places uneven, leaves (Fig. 1Q,R; Fig. 2J; data not shown). Light-grown YFP:D6PK and D6PK:GFP seedlings have shorter roots than wild-type seedlings, fewer lateral roots, and epinastic cotyledons; dark-grown seedlings have a severely shortened and thickened hypocotyl (Fig. 2C,F,H,J; data not shown). When we introduced the YFP:D6PK transgene into d6pk d6pk1 d6pk2 mutants, we found that the effect of YFP:D6PK overexpression is epistatic to the d6pk mutations and we therefore reasoned that the YFP:D6PK fusion protein is functional. Based on this observation, we restricted our further analysis to YFP:D6PK transgenic plants (data not shown).

Similarly to d6pk mutants, we found YFP:D6PK seedlings to have fewer emerged lateral roots (Fig. 2C,J). However, unlike the d6pk mutants, which are defective in lateral root initiation, YFP:D6PK seedlings initiate lateral roots but are defective in their outgrowth (Fig. 3C). Furthermore, we found YFP:D6PK overexpression to be sufficient to induce adventitious root formation in the hypocotyls of dark-grown seedlings (Fig. 3K,L), and to cause stronger DR5:GUS expression in leaves of YFP:D6PK plants, notably in the uneven leaf areas (Fig. 3L,J). At the same time, the DR5:GUS maximum was unaltered in the root tips of YFP:D6PK seedlings (Fig. 3C,G). In summary, these findings suggest that auxin response or auxin distribution are altered in YFP:D6PK plants. Because our auxin response experiments largely supported the notion that auxin responses are unaffected in YFP:D6PK plants (see Fig. S3A-C in the supplementary material) and because we found that YFP:D6PK seedlings are less sensitive to NPA (Fig. 3M), we reasoned that the YFP:D6PK phenotype is most likely to result primarily from altered auxin transport.

**D6PK and PIN proteins colocalize at the basal membrane of root cells**

Despite the fact that D6PK and its homologs are devoid of sequence motifs that would indicate that the proteins might reside in or at the plasma membrane, we found YFP:D6PK to localize to the basal (lower) membrane of various root cell types, specifically stele,
cortex, epidermis and lateral root cap cells (Fig. 4). In turn, the YFP:D6PK protein – although expressed from the constitutive 35SCaMV promoter – was not detectable in the root meristem or in columella root cap cells, suggesting that YFP:D6PK might be regulated at the posttranscriptional level (Fig. 4S).

The basal localization of YFP:D6PK in the stele is reminiscent of the basal localization of PIN auxin efflux facilitators. Using immunostaining, we could indeed show that YFP:D6PK colocalizes with basally localized PINs, specifically with PIN1 in stele cells (Fig. 4C), with PIN2 in cortex cells (Fig. 4K), and with PIN4 in lateral root cap cells (Fig. 4O), suggesting a possible functional relationship between these proteins in controlling auxin transport.

Unlike PID, which belongs to the same AGC kinase family as D6PK, and whose absence and presence had been shown to determine PIN polarity (Friml et al., 2004; Michniewicz et al., 2007), we found PIN polarity (as well as PIN abundance) to be unaltered in the roots of any of the D6PK genotypes examined (Fig. 4A-D,I-P). This was most obvious in the case of PIN2, which localizes to the basal membrane in cortex cells (C) and to the apical (upper) membrane in epidermal cells (E). In neither cell type did the presence or absence of D6PK affect the differential polarity of PIN2 (Fig. 4I-L). We therefore reasoned that neither changes in PIN polarity nor changes in PIN abundance are the likely cause of the reduced auxin transport in d6pk mutants.

Next, we examined the recycling of PIN1 and YFP:D6PK in response to auxin and the fungal toxin Brefeldin A (BFA). Auxin was shown to inhibit PIN1 endocytosis (Paciorek et al., 2005), and BFA treatment is known to block the recycling of PINs to the plasma membrane and leads to the accumulation of PIN proteins in intracellular compartments (Geldner et al., 2003). We reasoned that D6PK might be important for PIN endocytosis or PIN recycling, and tested the effects of auxin and BFA in the d6pk mutants and in YFP:D6PK seedlings using previously described experimental conditions (Geldner et al., 2003; Paciorek et al., 2005). However,
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Fig. 4. YFP:D6PK colocalizes with PIN proteins at the basal membrane of root cells. (A-D) Immunostaining with anti-PIN1 (red signal) reveals the lower (basal) localization of PIN1 in stele cells of roots of wild-type, d6pk mutant and YFP:D6PK transgenic seedlings (red arrowheads). YFP:D6PK (green signal) and PIN1 (red signal) colocalize at the basal membrane of stele cells (green and red arrowheads). Spearman's correlation coefficient for the PIN1 and YFP:D6PK signal shown in D is 0.586. (E-H) Following treatment with BFA, PIN1 (red) accumulates in endosomal compartments in all three genotypes (red arrowheads). YFP:D6PK (green) looses its association with the membrane and becomes cytoplasmic following BFA treatment (G,H). Spearman's correlation coefficient for the PIN1 and YFP:D6PK signal shown in H is 0.0135. (I,J) Immunostaining with anti-PIN2 (red) reveals the apical localization of PIN2 in root epidermal cells and the basal localization of PIN2 in cortical cells (red arrowheads). YFP:D6PK (green) colocalizes with basal PIN2 in the cortex but not with apical PIN2 in the epidermis (green and red arrowheads; K,L). E, epidermis; C, cortex. Spearman's correlation coefficient for the PIN2 and YFP:D6PK signal in K, in cortical cells is 0.255, in epidermal cells is -0.609. (M-P) Immunostaining with anti-PIN4 (red) reveals the basal localization of PIN4 in cells of the root tips of wild-type, d6pk mutant and YFP:D6PK transgenic seedlings. YFP:D6PK (green) and PIN4 (red) colocalize at the basal membrane of lateral root cap cells (green and red arrowheads; O,P). Spearman's correlation coefficient for the PIN4 and YFP:D6PK signal shown in P is 0.558. (Q-S) Propidium iodide staining of wild-type, d6pk d6pkl1 d6pkl2 mutant and YFP:D6PK roots reveals that the different genotypes have normal root morphology. (S) Although overexpressed, YFP:D6PK (green, live imaging) is not detectable in the central root meristem, but it is expressed in lateral root cap cells, cortical cells, the epidermis and stele cells (see also C,D). Our experiments did not reveal any alterations in the behavior of PIN1 in the d6pk mutants or in YFP:D6PK plants (Fig. 4E-H; data not shown). We therefore consider it unlikely that D6PKs exert their role in auxin transport by controlling PIN1 endocytosis or recycling.

Following BFA treatment, we made the interesting observation that YFP:D6PK looses its affinity for the plasma membrane. However, YFP:D6PK did not accumulate with PIN1 in BFA-induced compartments but was dispersed throughout the cytoplasm (Fig. 4E-H). This observation suggests that YFP:D6PK is retained at the plasma membrane by a BFA-sensitive component. Because PIN1 represents a good candidate for such a BFA-sensitive interaction partner, we introduced YFP:D6PK in a pin1 mutant background. We found, however, that YFP:D6PK is still plasma-membrane associated in a pin1 mutant (data not shown), and we therefore presently rule out that PIN1 is the protein required for the membrane association of YFP:D6PK. In addition, BFA treatments also revealed a differential growth response between the wild type and the D6PK genotypes. We found that BFA-induced agravitropic growth occurs in d6pk mutants at lower concentrations than in the wild type, and at the same time we found YFP:D6PK seedlings to be less sensitive to BFA (see Fig. S4 in the supplementary material). This demonstrates that altering D6PK dosage affects the BFA sensitivity of Arabidopsis roots with regard to root gravitropism, which is an auxin transport-dependent mechanism.

PIN proteins are in vitro and in vivo phosphorylation substrates of D6PK

We next examined whether PIN proteins are phosphorylation substrates of D6PK. To this end, we expressed and purified the cytoplasmic domain (cytoplasmic loop) of PIN1, PIN2, PIN3, PIN4 and PIN7 as a poly-histidine-tagged peptide from bacteria, and used the purified proteins as in vitro phosphorylation substrates for recombinant purified GST-tagged D6PK (Fig. 5A). Interestingly, each of the PIN protein fragments was efficiently phosphorylated by GST:D6PK, but not by a kinase-dead GST:D6PKin variant. Recombinant purified GST:PIN1 was also phosphorylated by YFP:D6PK, but not by YFP:D6PKin, after immunoprecipitation of the D6PK fusion proteins from transgenic plants (Fig. 5B). Finally, the overexpression of YFP:D6PK or of untagged D6PK (but not of YFP:D6PKin) led to the phosphorylation of a functional PIN1::GFP fusion protein in transiently transformed Arabidopsis protoplasts (Fig. 5C). Taken together, these data suggest that PIN proteins are phosphorylation targets of D6PKs in planta, and this finding invites the conclusion that D6PK and PIN proteins functionally interact. The phosphorylation reactions outlined above were not affected when the material was treated with IAA or NPA, or when the compounds were included in the phosphorylation reactions, which suggests that IAA and NPA do not directly modulate D6PK activity.

Next, we tested the genetic interaction between D6PK genes and PIN1 by introducing d6pk mutations into a pin1 mutant background. In support of the postulated interaction between D6PK and PIN1, we found that the pin1 mutant phenotype is enhanced in the presence of the d6pk d6pkl1 d6pkl2 mutations and the d6pk d6pkl2 mutations, the latter of which does not have a phenotype on its own: the pin1 d6pk d6pkl1 d6pkl2 and the pin1 d6pk d6pkl2 mutants were significantly smaller than the respective pin1 or d6pk mutants, and the leaves of pin1 d6pk d6pkl2 were significantly shorter than those of pin1 and d6pk d6pkl2 mutants, respectively (Fig. 5C; see Table S1 in the supplementary material). Because these findings indicated an important role for D6PKs as regulators of PIN1, and because undifferentiated pin-formed shoot apices are a hallmark phenotype of pin1 mutants, we examined the d6pk mutants for similar defects.
Following a more careful analysis, we found that the d6pk d6pkl1 d6pkl2 d6pkl3 quadruple mutant frequently develops axillary shoots with differentiation defects that were strongly reminiscent of the differentiation defects of primary and axillary pin1 mutant shoots (Fig. 1N-P). We thus propose that D6PKs are regulators of auxin transport, which might, at least in part, act together with PIN auxin efflux facilitators.

**DISCUSSION**

In the present study, we have characterized four highly homologous members of the Arabidopsis AGCVIIIa kinase family, which we designated the D6PKs (see Fig. S1 in the supplementary material). Based on the phenotypes of the d6pk mutants and the YFP:D6PK overexpressing lines, we judge that these kinases have redundant functions in regulating lateral root initiation, gravitropism, and shoot differentiation in axillary shoots. In addition, we could show that auxin transport is impaired in d6pk mutants, that DR5::GUS expression is altered in d6pk mutant root tips, and that DR5::GUS expression is absent at the presumed sites of lateral root initiation in d6pk mutants. It is known that many of these defects can be mimicked in wild-type plants by treatment with the auxin efflux inhibitor NPA. Based on our findings and the phenotypic similarities between d6pk mutants and NPA-treated plants, we suggest that D6PKs regulate a protein that is close to the site of action of NPA. Because D6PK and the PIN auxin efflux facilitators colocalize in planta, and because D6PK phosphorylates PINs, our data also invite the hypothesis that D6PKs interact with PIN proteins and possibly regulate their function by phosphorylation. Although we present several lines of evidence that include a functional link between D6PK and PIN, including the phosphorylation of PINs by D6PK, we are at present unable to shed light on the mechanistic interplay between the two protein families. Based on our cell biological analyses, we can rule out, however, that D6PKs control PINs by changing their abundance, tissue distribution, polarity, endocytosis or recycling.

We have been unable to detect any effects of auxin or NPA on the expression, abundance, localization or biochemical activity of D6PK. In this respect, it is noteworthy that we found that several D6PK genes are expressed strongly at the sites of lateral root initiation but that the expression of none of the D6PK genes is induced by auxin alone. Furthermore, D6PK expression appears to be regulated at the posttranscriptional level because YFP:D6PK — although overexpressed — is not detected in the root meristem or the columella root cap. Regarding YFP:D6PK polarity, we also would like to stress the fact that, although D6PK and PINs colocalize at the basal membrane of several other root cell types, D6PK and PIN2 do not colocalize in root epidermal cells, where PIN2 is the most abundant PIN. Thus, at least in epidermal cells, the polar distribution of D6PK is regulated independently from that of PIN2. Taking these findings together, we propose that D6PK regulates auxin efflux efficiency and that the biological activity D6PK is, at least in part, determined by its polarity and by its expression domain. Based on the observed interaction between D6PK and PINs, it could be speculated that D6PKs enhance the PIN auxin efflux facilitator function by phosphorylation or by another mechanism, e.g. by influencing their ability to interact with other proteins required for efficient auxin transport, such as the MDR/PGPs. The regulatory mechanism responsible for altered auxin transport in d6pk mutants is one important question that needs to be addressed in future experiments.

For several reasons, we have focused in the present study on the PIN proteins as putative phosphorylation targets of D6PK. First, we observed a number of morphological phenotypes in d6pk mutants that have also been observed in pin mutants (Benkova et al., 2003; Chen et al., 1998; Friml et al., 2002b; Galweiler et al., 1998; Luschnig et al.,...
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Supplementary material

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References


