The polarly localized D6 PROTEIN KINASE is required for efficient auxin transport in 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 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 Arabidopsis root cells, where it colocalizes with PIN1, PIN2 and PIN4. 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: Arabidopsis, Protein kinase, Auxin transport, PIN proteins, Lateral root

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 (Bainbridge 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; Swalup et al., 2001; Wisniewska et al., 2006). Mutations in AUX1/LAX genes have been reported to affect gravitropism, lateral root formation, and phyllotaxy (Bainbridge et al., 2008; Bennett et al., 1996; Marchant et al., 2002; Marchant et al., 1999; Swalup et al., 2008). Mutations in single 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 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, pin mutant phenotypes can in many cases be mimicked by the application of the auxin efflux inhibitor naphthylphtalamic 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). PID overexpression leads to a shift in the polarity of at least PIN1, PIN2 and PIN4 from the basal (lower) to the apical (upper) membrane in root cortex and lateral root cap cells (Friml et al., 2003). These PID-dependent polarity changes can be reverted by increasing the expression of PP2A, which suggests that PIN polarity and auxin efflux are at least in part controlled by 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 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/t-dnaexpress) and obtained from the Nottingham Arabidopsis Stock Centre: d6pk-1 (SALK_061847), d6pk-2 (SAIL_242_C05), d6pk1-1 (SALK_056618), d6pk2-1 (SALK_005798), d6pk1-2 (SALK_086127), d6pk3-1 (SALK_011507), d6pk3-2 (SALK_047347) and d6pk3-3 (SALK_081961). The pin1 (SALK_097144) allele was identified and obtained in the same manner. The alleles d6pk-1, d6pk1-1, d6pk2-1 and d6pk3-2 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 d6pk1/d6pk1 d6pk2/D6PKL2 and YFP:DPK 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 or D6PK-RV-GW2 into pBGWFS7 (Karimi et al., 2002) and transformed into Arabidopsis thaliana (Columbia) genomic DNA (primer details can be provided on request). The fragments were cloned into the Gateway-compatible vector pEXTAG-YFP-GW (a gift from Jane Parker, Cologne, Germany). 3SS: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 p35SGW-MYC and MDC83 (Curtis and Grossniklaus, 2003). YFP:D6PK, D6PK-GFP and 3SS: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 one reported here for YFP:D6PK. YFP:D6PK was introgressed into the d6pk/d6pk d6pk1/d6pk1 d6pk2 mutant and YFP:D6PK overexpression was found to overcome the d6pk/d6pk d6pk1/d6pk1 phenotype, suggesting that YFP:D6PK encodes a functional D6PK. YFP:D6PK contains a K to E amino acid substitution in the ATP-binding pocket of the kinase and was obtained by PCR-based mutagenesis (Sawano and Miyawaki, 2000) of YFP:D6PK with the primer D6Pkin. D6PK promoter fragments corresponding to a 2-3 kb region upstream of the ATG start codon were amplified from Arabidopsis thaliana (Columbia) genomic DNA (primer details can be provided on request). The fragments were cloned into the Gateway-compatible vector pBGWFS7 (Karimi et al., 2002) and transformed into Arabidopsis thaliana (Columbia).

The cytoplasmic loops of PIN1, PIN2, PIN3, PIN4 and PIN7 were identified based on homology to a previously published prediction of PIN topology (Muller et al., 1998). The corresponding gene fragments were amplified by RT-PCR with specific PINLOOP-FW and PINLOOP-RV primers from Arabidopsis thaliana (Columbia) mRNA. The Gateway-system compatible fragments were then cloned into the expression vectors pDEST17 (Invitrogen, Carlsbad, CA, USA), to generate constructs for the bacterial expression of His-tagged PINLOOP fusion proteins, namely HIS:PIN1 through HIS:PIN7, and pDEST15 (Invitrogen, Carlsbad, CA, USA), to generate GST:PIN1. GST:D6PK was obtained by insertion of the D6PK open reading frame amplified with D6PK-FW-GW3 and D6PK-RV-GW3 into pDEST15. The GST:D6PKin kinase-dead variant was derived from GST:D6PK with the primer D6Pkin (Sawano and Miyawaki, 2000).

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:dH2O:glycerol (20:9:3) and examined using an Axiohot 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 d6pk1 mutants and placed in inverted orientation for one hour in 30 μl auxin transport buffer [500 pM 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 kBaq (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 Hydroxyle 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 on RNA extracted from 7-day-old light-grown Arabidopsis seedlings with the RNAeasy 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 10 mM 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:dH2O:Glycerol (20:9:3) and examined using an Axiohot (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 (15 mg fresh weight) were ground in 1 ml pre-chilled (–20°C) 80% methanol containing 1% acetic acid (v/v). The sample was extracted for 2 hours at 4°C under continuous shaking. As an internal standard, 150 pg of d2-IAA (Sigma-Aldrich, Oakville, ON, Canada) was added. After centrifugation (10,000 μg) was reverse transcribed with an oligo-dT primer using M-MuLV Reverse Transcriptase (Fermentas, St Leon, Germany). 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.
spectrometer/mass spectrometer (LC-ESI-MS/MS). IAA was quantified on an LC (Acquity Ultra Performance LC, Waters, Milford, MA)-MS/MS (Q-TOF Premier, Micromass Technologies, Manchester, UK) system using an Acquity UPLC BEH C18 column (Waters, Milford, MA, USA). A binary solvent system was used consisting of H2O as solvent A and acetonitrile containing 0.05% acetic acid as solvent B. Separations were performed using an isocratic elution with 15% solvent B at a flow rate of 0.2 ml/min. The retention time of IAA and d2-IAA was 6.67 minutes. The MS/MS conditions were as follows: capillary, 2.6 kV; source temperature, 80°C; desolvation temperature, 400°C; cone gas flow 0 l/hour; desolvation gas flow, 500 l/hour; collision energy, 8.0; MS/MS transition, (m/z) 176/130 (unlabeled IAA), 178/132 (d2-IAA). The levels of IAA were determined against a calibration curve, which was obtained by injecting a series of standard solutions that contained a fixed concentration of d2-IAA (100 pg/ml) and varying concentrations of unlabeled IAA. MassLynx software 4.1 (Waters, Manchester, UK) was used to calculate IAA concentrations from the LC-MS-MS data.

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). Spearmann’s correlation coefficients were calculated using the Imagej plugin from http://www.cibp.ac.uk/~afrench/coloc.html, according to French et al. (2008).

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 Oftlinga, 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 (PVPK-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, d6pkl2-2 and d6pkl3-2 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 d6pkl double or the d6pk d6pkl1 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 agravitropic (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, agravitropic root growth, and reduced lateral root formation are phenotypes that are frequently observed in auxin response or transport mutants (Chen et al., 1998; Draymasiri 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) (Benkovka 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 (Casimirol 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.
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

Fig. 1. Isolation of d6pk mutants and YFP:D6PK overexpression lines. (A) Schematic representation of the four Arabidopsis D6PK genes, names of the mutant alleles, and positions of the respective T-DNA insertions. Black triangles mark the positions of the T-DNA insertions in lines that were used for mutant analyses. Introns are represented by lines, protein-coding exon sequences are presented as black boxes, non-coding exon sequences as gray boxes. (B) The RT-PCR-based gene expression analysis (28 PCR amplification cycles) of D6PK gene expression in the mutant alleles used for this study reveals the absence of full-length transcripts in d6pk-1, d6pkl2-2 and d6pkl3-2, and reduced transcript levels in d6pkl1-1. (C-M) Phenotype of 6-week-old adult plants (top) and vegetative rosettes of 3-week-old plants (bottom) of d6pk single, double, triple and quadruple mutants, as indicated. Scale bars: 6 cm. (N-P) The differentiation defects of apical meristems from lateral shoots of d6pk quadruple mutants (O) are reminiscent of the differentiation defects observed in pin1 mutant alleles (P). Scale bar: 0.5 cm. (Q) The overexpression of YFP:D6PK results in plants with reduced plant height and broader leaves, when compared with wild type. Scale bar: 6 cm. (R) In contrast to wild-type leaves, the leaves of YFP:D6PK plants are shorter and broader, and in some places are uneven. Scale bar: 1 cm.
NPA, we found that d6pk d6pkl1 d6pkl2 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 d6pkl1 d6pkl2 mutant stem segments using radiolabeled IAA, we revealed a strong decrease in IAA transport in the d6pk d6pkl1 d6pkl2 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 d6pkl1 d6pkl2 mutant (14.3±3.1 ng IAA/g fresh weight; n=6) seedlings, we concluded that the d6pk d6pkl1 d6pkl2 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; 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,I; data not shown). When we introduced the YFP:D6PK transgene into d6pk d6pkl1 d6pkl2 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. 3J). 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 stelle,
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,
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 stelle 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 stelle 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 d6pk1 d6pk2 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 stelle 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 d6pk1 d6pk2 mutations and the d6pk d6pk12 mutations, the latter of which does not have a phenotype on its own: the pin1 d6pk d6pk1 d6pk2 mutations and the d6pk d6pk12 mutations were significantly smaller than the respective pin1 or d6pk mutants, and the leaves of pin1 d6pk d6pk12 were significantly shorter than those of pin1 and d6pk d6pk12 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 $d6pk$ mutants frequently develop axillary shoots with differentiation defects that were strongly reminiscent of the quadruple mutant. 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 roots, 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 basolateral 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 an 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.,...
1998; Muller et al., 1998; Scarpella et al., 2006), and these defects appear generally to be stronger—as far as can be judged from published work—than those observed in mutants deficient in all four AUX1/LAX genes (Bainbridge et al., 2008; Casimiro et al., 2001; Marchant et al., 2002; Marchant et al., 1999) (Figs 1, 2). Second, we observed a shift of the DR5:GUS expression maximum in the root tips of d6pk mutants, and similar changes in DR5:GUS activity were also reported in pin2 and pin4 mutants (Friml et al., 2002a; Sabatini et al., 1999). Third, we observed a synergistic genetic interaction between PIN1 and the D6PK genes, thereby supporting the idea that the two proteins act in close proximity. Finally, D6PKs belong to the same protein kinase family as PID, which phosphorylates PIN1 and whose mutant phenocopies the pin1 mutant phenotype (Michniewicz et al., 2007). Therefore, PINs are good candidates for D6PK phosphorylation substrates. However, it may be that the observed D6PK-PIN interactions are not functionally relevant in vivo or that other auxin transport proteins are D6PK substrates. In view of the fact that YFP:D6PK localizes to the basal membrane of root epidermal cells, where it does not colocalize with PIN2, D6PK may well have additional phosphorylation targets.

Because PID and D6PKs all belong to the AGCVIIIa kinase family and because all are functionally implicated in regulating PIN activity, it may be reasoned that these kinases regulate auxin transport in the same manner (Galvan-A Cuadra and Offringa, 2007; Michniewicz et al., 2007). In contrast to what was observed for PID loss-of-function and overexpression lines, our data for D6PK show that PIN polarity is unaltered in d6pk mutants, as well as in YFP:D6PK overexpression lines. We therefore suggest that PID and the D6PK proteins do not have a redundant function. A differential role of D6PK and PID is also supported by the fact that the overexpression of either kinase has a different effect on plant growth. First, YFP:D6PK seedlings are defective in lateral root formation and the development of dark-grown YFP:D6PK seedlings is strongly impaired. In turn, lateral root development is unaffected in PID overexpression lines and dark-grown seedlings have only subtle defects (Benjamin et al., 2001; Friml et al., 2004). Second, whereas the roots of PID overexpressing seedlings grow gravitropically and their meristems collapse, YFP:D6PK overexpression lines do not grow gravitropically and they have an intact meristem and normal DR5:GUS reporter activity (Benjamin et al., 2001; Friml et al., 2004). Another difference between D6PK and PID resides in the polar localization of the proteins. Whereas YFP:D6PK (as well as D6PK:GFP) localize to the basal membrane of all root cell types examined, including epidermal cells, PID:YFP and PID:GFP have an apicobasal localization in the epidermis (Galvan-Ampudia and Offringa, 2007; Michniewicz et al., 2007). Taken together, these differences indicate that D6PKs and PID have differential biochemical functions and control plant growth in a different manner. Nevertheless, our observations reported here, together with the observations previously reported for PID, could help to reveal the molecular mechanisms that underlie the functions of other AGCVIIIa kinases, such as PHOT and WAG protein kinases.

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Supplementary material
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