A protein kinase target of a PDK1 signalling pathway is involved in root hair growth in *Arabidopsis*

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Here we report on a lipid-signalling pathway in plants that is downstream of phosphatidic acid and involves the *Arabidopsis* protein kinase, AGC2-1, regulated by the 3′-phosphoinositide-dependent kinase-1 (AtPDK1). AGC2-1 specifically interacts with AtPDK1 through a conserved C-terminal hydrophobic motif that leads to its phosphorylation and activation, whereas inhibition of AtPDK1 expression by RNA interference abolishes AGC2-1 activity. Phosphatidic acid specifically binds to AtPDK1 and stimulates AGC2-1 in an AtPDK1-dependent manner. AtPDK1 is ubiquitously expressed in all plant tissues, whereas expression of AGC2-1 is abundant in fast-growing organs and dividing cells, and activated during re-entry of cells into the cell cycle after sugar starvation-induced G1-phase arrest. Plant hormones, auxin and cytokinin, synergistically activate the AtPDK1-regulated AGC2-1 kinase, indicative of a role in growth and cell division. Cellular localisation of GFP-AGC2-1 fusion protein is highly dynamic in root hairs and at some stages confined to root hair tips and to nuclei. The *agc2-1* knockout mutation results in a reduction of root hair length, suggesting a role for AGC2-1 in root hair growth and development.

Introduction

Lipid-derived signalling molecules play a central role in regulation of growth responses to a wide range of hormonal, environmental and developmental signals in fungi, plants and animals (Vanhaesebroeck *et al*, 2001; Meijer and Munnik, 2003). Although plants produce many signalling lipids known in animals, with the important exception of phosphatidylinositol (3,4,5) triphosphate, the protein targets and signalling pathways of plant phospholipids are largely unknown (Mueller-Roeber and Pical, 2002).

In animal cells, PDK1 is a central integrator for signalling events downstream of various receptors that stimulate the PI 3-kinase, and thus regulates processes, among which the most prevalent is the maintenance of balance between growth, cell division and apoptosis in animals (Alessi, 2001). So far, it is uncertain as to how lipids regulate PDK1 activity and modulate its localisation, activation, stability, or a combination of these (Storz and Toker, 2002). Recently, a functional homologue of animal PDK1 was identified in *Arabidopsis*, named AtPDK1, that was demonstrated to complement PDK1 mutants, as well as to activate animal PKB *in vitro* (Deak *et al*, 1999). These data suggest that downstream targets of PDK1 may be similar in yeast, plant and animal cells. In comparison to yeast and animal PDK1s, the PH domain of *Arabidopsis* and rice PDK1 kinases lack two conserved amino-acid residues that are required for high-affinity binding to PI(3,4,5)P₃. In addition, the PH domain of *Arabidopsis* PDK1 was shown to bind a wide spectrum of lipids (Deak *et al*, 1999), suggesting that lipid activators of the plant PDK1 kinases could significantly differ from those of their animal orthologues.

In animals, the primary targets of PDK1 are a class of protein kinases, which are closely related to the cAMP-dependent (PKA) and cGMP-dependent protein kinases, and protein kinase C (PKC), collectively called AGC kinases (Storz and Toker, 2002). Activation of all members of the AGC kinase family occurs by phosphorylation of a Thr or Ser residue that lies in a region of kinase catalytic domain termed the T-loop. In selected members, including ribosomal S6 kinase, protein kinase B, various isoforms of PKC and serum and glucocorticoid-inducible kinase, the T-loop phosphorylation is carried out by PDK1 (Biondi and Nebreda, 2003).

Although there have been considerable efforts to find and characterise PKA- or PKC-related activities in plants, the available results are so far controversial (Bogre *et al*, 2003). S6K is a member of the *Arabidopsis* AGC kinase family (Turck *et al*, 1998), but its role in phospholipid signalling and its interaction with AtPDK1 remain to be established. By screening for AtPDK1 interacting partners in the yeast two-hybrid system, we have identified two members of the *Arabidopsis* AGC kinase family, which we call AGC1-1 and AGC2-1. Here we show that AtPDK1 directly regulates AGC2-1 and that phosphatidic acid is the lipid activator of this AtPDK1 signalling pathway.

Results

AtPDK1 interacts with members of the AGC kinase family

To search for potential components of the AtPDK1 signalling pathway, a yeast two-hybrid screen was carried out using

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full-length AtPDK1 as bait and a pACT2 prey cDNA library prepared from cultured *Arabidopsis* cells (Nemeth *et al*, 1998). By screening 1.2×10^6 transformed yeast colonies, 24 cDNA clones encoding AtPDK1 interacting factors were identified, which were sorted into 11 groups. Two of these prey cDNAs encoded Ser/Thr protein kinases, AGC1-1 (At5g55910) and AGC2-1 (At3g25250), that carried conserved domains of the AGC kinase family, which is represented in *Arabidopsis* by 39 members. In a recent review we follow a nomenclature for the AGC kinase family in *Arabidopsis* that provides working names for all members but incorporate and keep published names wherever available (http:// www.arabidopsis.org/info/genefamily/AGC.html; Bogre *et al*, 2003). There are two closely related PDK1 genes in *Arabidopsis*, PDK1-1 and PDK1-2, sharing 93% amino-acid identities. PDK1-1 is identical to the originally named AtPDK1, the name we use within this paper (Deak *et al*, 1999). AGC1-1 and AGC2-1 share 38% sequence identity and show a significant similarity (31% identical) to mouse PKA (accession number PO5132) over the entire coding region.

Interaction of AGC1-1 and AGC2-2 with AtPDK1 requires a C-terminal hydrophobic motif

Arabidopsis AtPDK1-1 and its analogue AtPDK1-2 interacted with AGC1-1 and AGC2-1 in yeast two-hybrid assays (Figure 1A). AGC2-1 appeared to recognise the AtPDK1 kinase domain, as no interaction could be detected with the PH domain of AtPDK1 (Figure 1A). PDK1 binding to selected AGC kinases in animals, such as to S6K, PKB, aPKC or to SGK, has been shown to require a C-terminal hydrophobic FXXF motif, called PDK1 interacting fragment (PIF) (Biondi and Nebreda, 2003). We generated two modified kinase constructs by exchanging the two conserved phenylalanines to alanine within the FXXF motif (M1), or replacing the last six C-terminal amino acids with an unrelated sequence, RGSGC (M2) (Figure 1B). Both mutated versions of AGC1-1 and AGC2-1 were unable to interact with PDK1 (Figure 1A). These data support the finding that both phenylalanines in the PDK1 interacting fragment (PIF) domain are required for interaction.

AGC1-1 and AGC2-1 share substrate specificities with protein kinase A

To determine the enzymatic activity and substrate specificities of AGC1-1 and AGC2-1 kinases, we performed *in vitro* kinase assays with different synthetic peptides known to be phosphorylated by animal AGC kinases (Obata *et al*, 2000). Both *Arabidopsis* AGC kinases were labelled with N-terminal haemagglutinin (HA) epitope tags, expressed in transfected *Arabidopsis* cells, and immunopurified for kinase assays 1 day after transfection using immobilised monoclonal anti-HA IgG. The kemptide peptide, which is readily phosphorylated by animal PKA (Kemp *et al*, 1977), was found to be the best substrate for both AGC1-1 and AGC2-1 (Figure 1C), which showed no significant difference in their substrate preference in these assays. To further characterise the substrate specificity of AGC1-1 and AGC2-1, we have used a kinase inhibitor peptide derived from the PKA inhibitor protein PKI, which binds to the catalytic pocket of animal PKA (Baude *et al*, 1994). The PKI peptide inhibited similarly the phosphorylation activities of HA-AGC1-1 and HA-AGC2-1 kinases with all substrate peptides tested (Figure 1C). These results
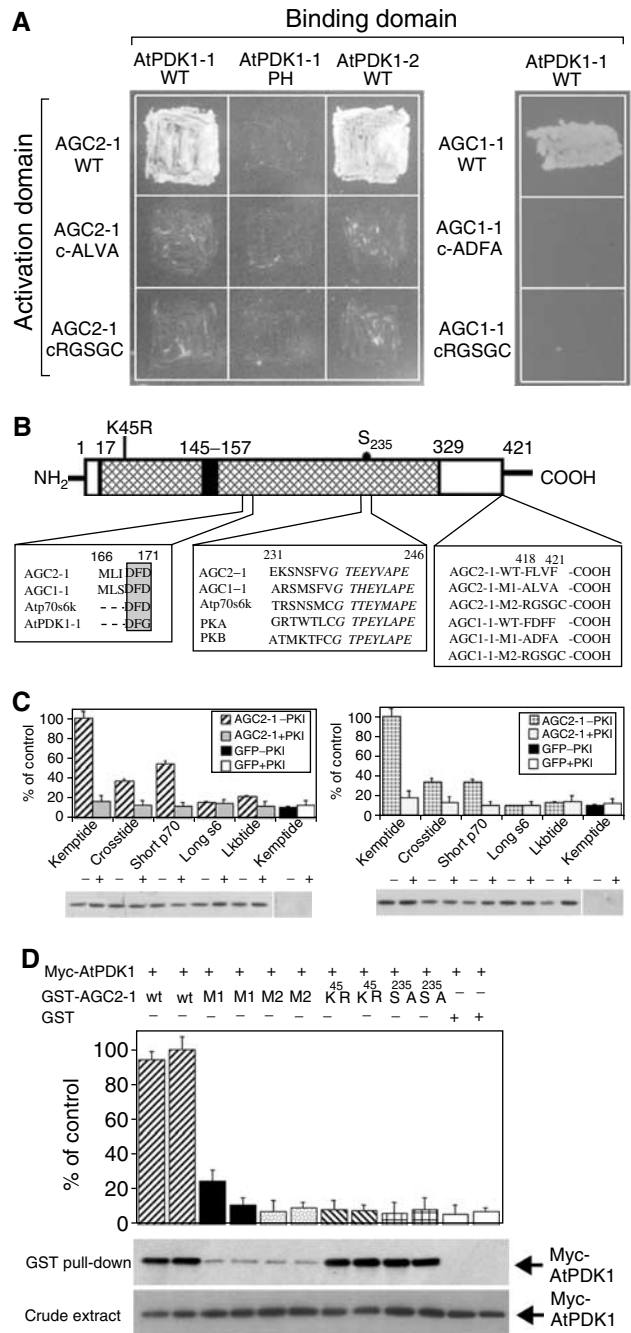
Figure 1 Interaction of AtPDK1 with AGC2-1 and AGC1-1. (**A**) Yeast two-hybrid interaction assay with wild-type AtPDK1-1 or AtPDK1-2 and with the pleckstrin homology (PH) domain of AtPDK1-1 fused to the Gal4 DNA-binding domain, and the wild-type AGC2-1 or AGC1-1 or their indicated C-terminal mutations fused to the Gal4 activation domain. (**B**) Schematic diagram showing the domain structure of AGC2-1 with alignments to AGC1-1, to Atp70S6K and to AtPDK1-1, and the site-directed mutations. Hatched box = catalytic domain; black box = ser/thr protein kinase active site signature. Conserved PDK1 phosphorylation site is underlined. (**C**) AGC1-1 and AGC2-1 substrate specificities against the indicated synthetic peptides in the presence or absence of the PKI inhibitory peptide. Activities are expressed as a percentage of the kemptide phosphorylation. In all cases, the AGC1-1 and AGC2-1 protein levels were determined by Western blotting using the HA antibody. (**D**) Interaction and activity of wild-type and mutant versions of GST-AGC2-1 when coexpressed with Myc-AtPDK1 in *Arabidopsis* protoplasts. Two independent experiments are shown. GST-AGC2-1 activity is expressed as a percentage of the wild type. Myc-AtPDK1 levels are shown in crude extracts or in GST pull-downs.

demonstrate that the substrate preferences of AGC1-1 and AGC2-1 resemble those of mammalian PKA enzymes.

The hydrophobic PIF domain of AGC2-1 is required both for AtPDK1 binding and for full activity

Next, we investigated whether interaction of AGC2-1 with AtPDK1 requires its hydrophobic PIF domain *in vivo* and how this interaction affects the enzyme activity of AGC2-1. For this, we cotransfected a Myc-tagged AtPDK1 kinase with GST-tagged AGC2-1 into protoplasts prepared from cultured *Arabidopsis* cells and tested for the presence of Myc-AtPDK1 in GST pull-down experiments, as compared to the input amounts in crude extracts. Protein kinase activity of AGC2-1 was determined using the kemptide peptide. These experiments confirmed that Myc-AtPDK1 is recruited by GST-AGC2-1 *in vivo* (Figure 1D). To answer the question whether the activity of AGC2-1 kinase is required for interaction with AtPDK1, we performed similar GST pull-down assays using a modified kinase, GST-AGC2-1 ($K^{45}R$). This inactivated kinase, in which a critical lysine residue required for phosphotransfer was changed to arginine, showed no change in its ability to interact with AtPDK1 (Figure 1D). To test whether the conserved AtPDK1 phosphorylation site on AGC2-1 at S^{235} within the activation loop is critical for AGC2-1 activity or its interaction with AtPDK1, we exchanged this serine residue to alanine ($S^{235}A$). While the activity of the mutated AGC2-1 was reduced to background levels, the phosphorylation site mutation had no effect on the AGC2-1 interaction with AtPDK1. These data showed that phosphorylation of AGC2-1 within the activation loop, possibly by AtPDK1, is critical for its activity, but not required for AtPDK1 interaction. Contrary to this, a 90–95% reduction in interaction was detected when the critical phenylalanine residues within the PIF (FXXF) domain were mutated (M1) or when the domain was exchanged as indicated in Figure 1B (M2). These mutant forms were also found to be inactive in kinase assays with kemptide as a substrate. These results indicated that either the association of AGC2-1 with AtPDK1 is required for its activity, or that the mutations within the PIF domain inactivated the kinase, as was previously found for PKA (Batkin *et al*, 2000). Interestingly, deletion of the C-terminal hydrophobic motif in PINOID, a representative of AGC1 kinase subgroup in *Arabidopsis* (Bogre *et al*, 2003), was observed to yield no change in its biological activity (Benjamins *et al*, 2001). Nonetheless, our results clearly showed that the C-terminal hydrophobic region of AGC2-1 is important for interaction with PDK1, and that phosphorylation of AGC2-1 at the activation site, possibly by AtPDK1, is required for its activity but not for its binding to AtPDK1.

AtPDK1 is an upstream activator of AGC2-1, but fails to activate AGC1-1

To address the question whether AtPDK1 mediates the activation of either AGC1-1 or AGC2-1 (or both), the activity of AGC kinases was monitored in cells, in which the expression of *AtPDK1* gene was inhibited using RNAi technology. The AtPDK1-RNAi construct was designed using the inverted hairpin-loop approach (Figure 2A). The efficiency of RNAi gene silencing was measured with the help of a cotransfected Myc-tagged AtPDK1 construct, the sequence of which was nearly identical to the endogenously expressed *AtPDK1* mRNA. The expression level of Myc-AtPDK1 reporter
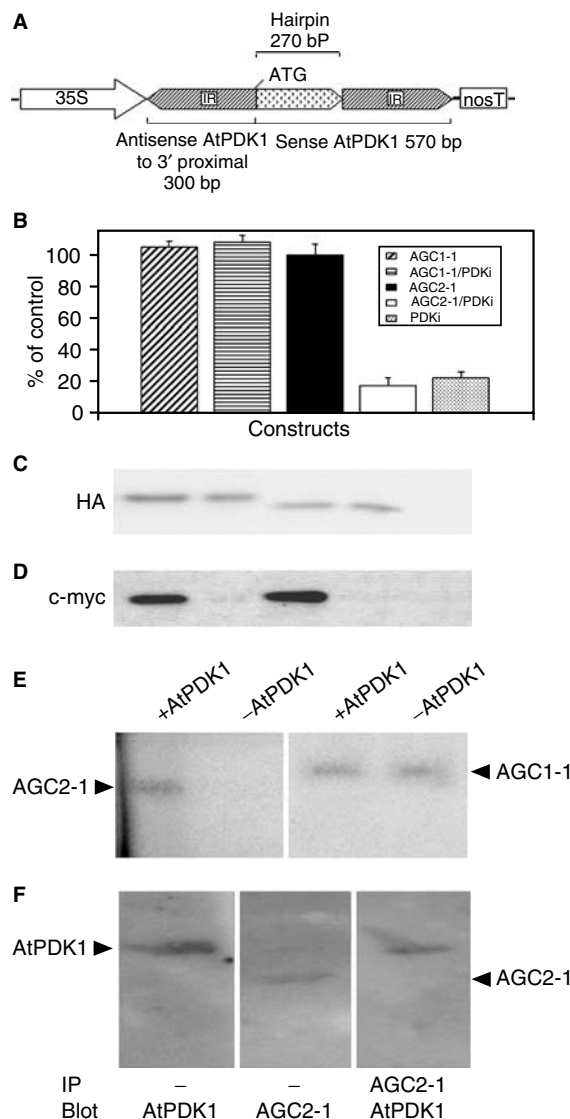
Figure 2 AGC2-1 but not AGC1-1 activity is dependent on AtPDK1 levels. (**A**) Schematic showing the AtPDK1-RNAi construct design. (**B**) AGC1-1 and AGC2-1 activities with and without the cotransfection of the AtPDK1-RNAi construct (PDKi) or the PDKi construct alone as a control. (**C**) Protein levels of AGC1-1 and AGC2-1 detected through the HA tag in the same samples as in (B). (**D**) Efficiency of the AtPDK1 RNA interference as determined by the protein levels of the Myc-AtPDK1 coexpressed within the same cells in the first four samples indicated in (B). (**E**) Phosphorylation of AGC1-1 and AGC2-1 by AtPDK1 *in vitro*. GST-tagged AGC1-1 and AGC2-1 were purified from transfected *Arabidopsis* cells under a noninduced inactive state and its phosphorylation was tested in a kinase reaction in the presence or absence of AtPDK1 and visualised by autoradiography. (**F**) Coimmunoprecipitation of endogenous AtPDK1 and AGC2-1. *Arabidopsis* cell lysate was tested for endogenous levels of AtPDK1 (lane 1) or AGC2-1 (lane 2) and for AtPDK1 in the AGC2-1 immunoprecipitate (lane 3) using AtPDK1- and AGC2-1-specific antibodies for immunoprecipitation (IP) and for Western blots (Blot) as indicated.

construct thus reflected the RNAi-mediated inhibition of endogeneous *AtPDK1* gene. The AtPDK1-RNAi construct reduced the expression of cotransfected Myc-AtPDK1 reporter to a nondetectable level (Figure 2D). This RNAi-directed ablation of AtPDK1 expression did not affect the activity of HA-AGC1-1, but reduced the kinase activity of HA-AGC2-1

kinase to a background level, which was comparable to the control, where no HA-AGC kinase was transfected into the cells (Figure 2B). The expression levels of HA-AGC1-1 and HA-AGC2-1 proteins were, however, not affected by cotransfection of the RNAi construct, as was revealed by immunoblotting with an anti-HA antibody (Figure 2C). These results showed that the presence of AtPDK1 was required for activation of AGC2-1, whereas AtPDK1 levels had no affect on AGC1-1 activity in dividing *Arabidopsis* cells.

AtPDK1 phosphorylates AGC2-1, but not AGC1-1
Next, we investigated whether AtPDK1 could phosphorylate directly the AGC kinases *in vitro*. Purified GST-AGC2-1 or GST-AGC1-1 were incubated with or without the purified Myc-tagged AtPDK1 in a protein kinase assay (Figure 2E). While AGC1-1 showed autophosphorylation in the absence of AtPDK1, AGC2-1 was only phosphorylated when coincubated with AtPDK1, suggesting an absolute requirement for AtPDK1 as upstream activating kinase.

AtPDK1 and AGC2-1 interact in vivo
We performed coimmunoprecipitation experiments to detect the interaction of endogenous AtPDK1 and AGC2-1 *in vivo* in *Arabidopsis* cells. We have raised antibodies against the AtPDK1 and AGC2-1 proteins. These anti-AtPDK1 and anti-AGC2-1 antibodies specifically reacted with AtPDK1 and AGC2-1 proteins, respectively, in Western blot analyses using cell lysates prepared from cultured *Arabidopsis* cells (Figure 2F, lanes 1 and 2). To detect *in vivo* association of these protein kinases, AGC2-1 was immunoprecipitated from *Arabidopsis* protein extracts using an immobilised polyclonal anti-AGC2-1 IgG and then the isolated immunocomplex was probed by Western blotting with an antibody raised against AtPDK1 (Figure 2F, lane 3). A specific band corresponding to AtPDK1 was detected in the AGC2-1 immunocomplex, which was not present with preimmune serum or with the IgG beads alone (data not shown). This confirmed *in vivo* the interaction of endogenous AtPDK1 and AGC2-1 proteins, and is in agreement with the results that we obtained with the yeast two-hybrid assay and with the GST pull-down experiments.

AtPDK1 activity is directly stimulated by phosphatidic acid and PI(4,5)P₂
We have previously observed that the PH domain of AtPDK1 directly binds PI3P and PA (Deak *et al*, 1999). To compare binding of PI3P and PA to AtPDK1, we have used so-called lipid-affinity matrices, which were prepared by covalent linkage of these lipids to affigel beads (Krugmann *et al*, 2002). As shown in Figure 3A, the PA beads retained considerably more GST-AtPDK1 than the PI3P beads. The specificity of this assay was confirmed using control lipid-binding domains of known lipid-binding specificity. These included the two PI3P-binding domains, PHOX domain of human p40[phox] protein (NCF-4, residues 1–148) (Kanai *et al*, 2001) and a double FYVE domain from mouse Hrs (residues 147–223) (Gillooly *et al*, 2000), and RPA, which is the PA-binding region of Raf-1 kinase (residues 390–426) (Rizzo *et al*, 2000). The FYVE and PHOX domains were retained on the PI3P beads, whereas the RAF-1 domain specifically bound to PA. Consequently, these results indicated that AtPDK1 preferentially binds PA.
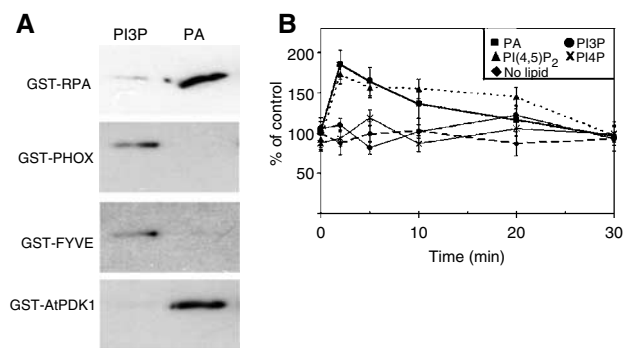

Figure 3 Binding specificity and activation of AtPDK1 by lipids. (**A**) N-terminally GST-tagged proteins of various lipid-binding domains were purified from *E. coli* and incubated with either PI3P or PA beads. The specifically bound proteins were eluted and detected by a GST antibody on Western blots. (**B**) AtPDK1 activity was determined against the synthetic peptide PIFtide. Phospholipids were added to cells for the indicated times and treatments labelled as follows: PA (solid line, filled square), PI(4,5)P₂ (dotted bold line, filled triangle), PI3P (solid line, filled circle), PI4P (solid line, cross), no lipid (dashed line, diamond). The activities of purified HA-AtPDK1, expressed in transfected protoplasts, were determined in triplicate.

Next, we tested whether external addition of various lipids to *Arabidopsis* cells resulted in altered AtPDK1 activity. First, we had to establish a protein kinase assay for AtPDK1. We found that AtPDK1 was able to phosphorylate directly a synthetic peptide, whose sequence encompasses the PDK1-docking site fused to the PDK1 phosphorylation site on the activation loop of the animal PKB, termed PIFtide (Biondi *et al*, 2000). As shown in Figure 3B, the exogenously applied PA very rapidly increased the AtPDK1 activity. Other negatively charged phospholipids, such as PI3P and PI4P, were not able to stimulate the activity of AtPDK1, with the exception of PI(4,5)P₂. These results showed that increased PA and PI(4,5)P₂ in cells are able to stimulate AtPDK1 activity. Whether this indicates a possible recruitment of AtPDK1 to membranes or other lipid compartments, however, remains to be established.

Phosphatidic acid enhances the activity of AGC2-1, but not AGC1-1, in an AtPDK1-dependent manner
Next, we tested whether the external addition of lipids to *Arabidopsis* cells could alter the activity of AGC kinases in an AtPDK1-dependent manner, which would indicate that phospholipid signalling is upstream of these kinases *in vivo*. As shown in Figure 4A, only PA increased the activity of AGC2-1, whereas none of the lipids tested could alter the activity of AGC1-1 (Figure 4B). PA-mediated activation of AGC2-1 was transient, showing an increase as early as 1 min. The peak level of AGC2-1 activity, occurring 10 min after stimulation, was approximately 70% higher than the basal activity measured in untreated dividing cells. When the cells were transfected with the AtPDK1-RNAi construct, not only was the PA activation abolished but the RNAi inhibition also resulted in a dramatic drop in basal activity, which decayed to background level throughout the time course (Figure 4C). These data showed that both the basal activity attained in cells cultured in hormone-containing medium and the PA-mediated induction specifically required the presence of AtPDK1.
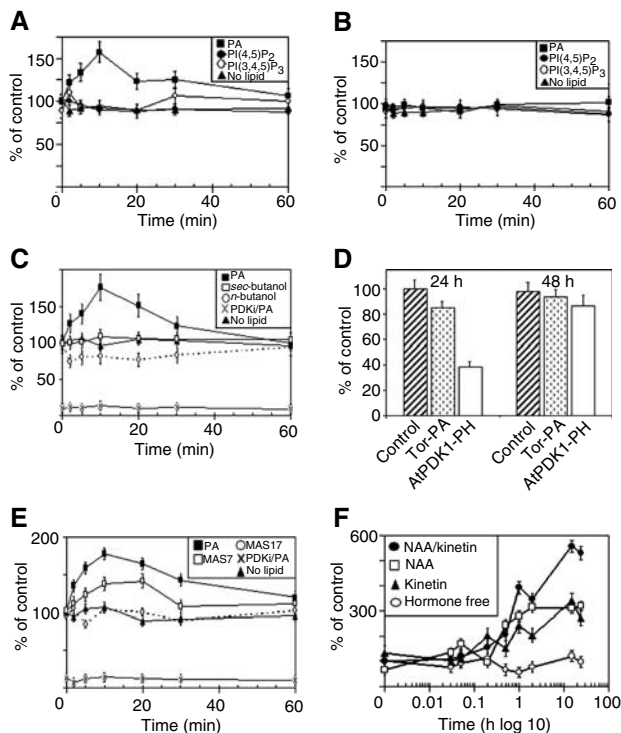
Figure 4 Regulation of AGC2-1 activity by phosphatidic acid and the plant hormones auxin and cytokinin. (**A**) Activity of HA-AGC2-1 that was expressed and then purified from *Arabidopsis* protoplasts treated with various lipids for the indicated times as follows: PA (solid line, filled square), PI(4,5)P_2 (solid line, filled circle), PI(3,4,5)P_3 (solid line, open circle), no lipid (solid line, filled triangle). (**B**) Activity of HA-AGC1-1 in an experiment as described in (A). (**C**) Phosphatidic acid specifically activates AGC2-1 through AtPDK1. The activity of HA-AGC2-1 in cells treated with PA (solid line, filled square), with 0.01% *n*-butanol to inhibit phosphatidic acid production (dotted line, open square), with 0.01% *sec*-butanol, as an inactive analogue (solid line, open square), or the control without treatment (solid line, filled square). AtPDK1 levels were ablated by expression of the PDKi construct as in Figure 2 in cells treated with PA (solid line, cross). (**D**) *Arabidopsis* cells were transfected with HA-AGC-2-1 and cotransfected either with the putative PA-binding domain of TOR fused to GFP (TOR-PA) or the PH domain of AtPDK1 fused to GFP (PDK1-PH), incubated for 24 or 48 h and the AGC2-1 activities were determined in triplicate. (**E**) Effects of the elevation of endogenous PA levels through activation of the heterotrimeric G-protein by mastoporan on AGC2-1 activity. Cells were treated for the indicated times with PA (solid line, filled square), 4 μM mastoporan analogue MAS7 (solid line, open square) or its inactive analogue Mas17 and with PA in cells where AtPDK1 levels were ablated by the cotransformation of the PDKi construct (solid line, cross) and compared to control without treatment (solid line, filled triangle). (**F**) Auxin and cytokinin synergistically elevate AGC2-1 activity. The hormones were added either singly or together (NAA = 0.5 mg/l, Kin = 0.05 mg/l), and HA-AGC2-1 activities were determined over a 24 h period. To control the expression of transfected constructs, in all experiments the HA-AGC1-1, HA-AGC2-1 or Myc-AtPDK1 levels were monitored by Western blot analysis of total cell lysates (data not shown).

In animal cells, PA signalling is produced either by the phospholipase C (PLC) pathway in combination with diacylglycerol kinase, or directly via phospholipase D (PLD). The latter pathway can specifically be inhibited by *n*-butanol, because PLD preferentially uses it as a *trans*-phosphatidylation substrate resulting in the production of phosphatidyl butanol (pBut) at the cost of PA. Therefore, *n*-butanol is widely used as a PLD inhibitor in plant and animal systems

(Munnik, 2001). In our assays, *n*-butanol treatment resulted only in a slight decrease of AGC2-1 activity. Some significance of this finding was suggested by the fact that a closely related compound, *sec*-butanol, which is not a substrate for PLD's transphosphatidylation activity (Munnik *et al*, 1995), had no effect on the activity of AGC2-1 (Figure 4C).

To modify endogenous PA levels and to assay whether PA binding to the PH domain of AtPDK1 can regulate the activity of AGC2-1, we tested whether we could titrate out the endogenous PA levels by overexpression of two PA-binding domains fused to GFP, the PH domain of AtPDK1 (AtPDK1-PH) and the putative PA-binding domain of AtTOR (TOR-PA), in *Arabidopsis* cells. As shown in Figure 4D, the activity of AGC2-1 kinase was reduced by about 50% 24 h after the expression of AtPDK1-PH. Following 48 h, the AGC2-1 activity was, however, reduced only to 87% of control, suggesting the induction of a compensatory mechanism. Overexpression of another PA-binding domain derived from the *Arabidopsis* TOR kinase (Menand *et al*, 2002) also reduced AGC2-1 activity, but only by 15% (Figure 4D).
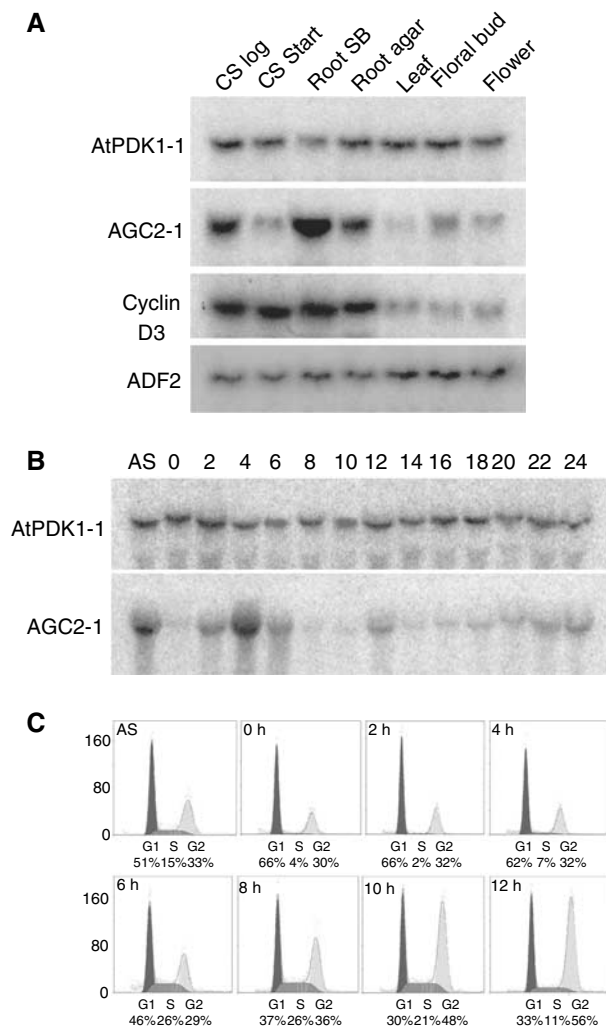
Mastoporan is a known activator of heterotrimeric G-proteins and triggers PA signalling via PLC and PLD activation (Munnik *et al*, 1995). This raised the question, whether mastoporan treatment could activate AGC2-1? We found a rapid and transient activation of AGC2-1 upon Mas7 treatment that was dose and time dependent. No activation was observed with Mas17, an inactive mastoporan analogue (Figure 4E). These data suggested that a G-protein-coupled receptor might operate upstream of AGC2-1. Taken together, these results strongly suggested that phosphatidic acid is involved in the activation of a signalling pathway upstream of AtPDK1 leading to subsequent AtPDK1-dependent activation of AGC2-1.

AGC2-1 activity is synergistically stimulated by the growth-promoting plant hormones auxin and cytokinin

Agonists that activate PDK1-related signalling pathways include growth factors and hormones in animal cells. Therefore, we have tested the ability of plant growth factors, auxin and cytokinin, to act as possible activators of AGC2-1. HA-tagged AGC2-1 was expressed by transfection in *Arabidopsis* cells and, 1 day later the cells were treated with auxin (napthylacetic acid 1 mg/l) and cytokinin (kinetin 0.2 mg/l). Cells were harvested at different time intervals, and the activity of AGC2-1 was determined after immunopurification with anti-HA IgG in kinase assays *in vitro*. When used separately, both auxin and cytokinin activated HA-AGC2-1 approximately 30 min after hormone application, leading to an activity peak about 10 h later. When auxin and cytokinin were added together, a synergistic stimulation of HA-AGC2-1 was observed (Figure 4F). The viability of cells 24 h after hormone starvation was confirmed by the inducibility of auxin-responsive promoters.

AGC2-1 mRNA is abundant in actively dividing tissues

To assess the tissue distribution of AtPDK1 and AGC2-1 mRNA expression, Northern blot analyses were performed. The AtPDK1 transcript was detected in all organs examined (Figure 5A). In contrast, steady-state levels of AGC2-1 mRNA were maximal in fast-growing roots and cultured cells, but low in nongrowing, fully developed leaves. This expression pattern of AGC2-1 resembled that of cyclin D3 in fast-growing

meristems (Gaudin *et al*, 2000). To examine this in more detail, we studied the expression pattern of *AGC2-1* gene in synchronised *Arabidopsis* cells. When sugar is withdrawn from the medium, a high proportion of cultured *Arabidopsis* cells is arrested in G1 phase, but re-enter cell cycle synchronously when sugar is added back (Menges and Murray, 2002). As shown in Figure 5C, sugar starvation in our experiments led to a dramatic decrease in the proportion of cells in S phase, but the number of cells in S phase increased again from 6 h onwards after re-feeding with sugar. In these synchronised cells, AtPDK1 was expressed evenly in all samples (Figure 5B). However, AGC2-1 expression was strongly induced when sugar was added back to the medium, showing a peak after 4 h, just before the cells entered S phase (Figure 5B). This correlation indicated that pathways controlling cell growth and re-entry to the cell cycle tightly regulate the expression of AGC2-1.

### Phenotypic characterisation of the agc2-1 mutant

To dissect the function of AGC2-1 genetically, we have identified a T-DNA-induced knockout mutation. A single insertion was located in the first intron and resulted in full inactivation of the *AGC2-1* gene (Supplementary Figure 1). For phenotypic characterisation of the *agc2-1* mutant, we first focused our attention on the analysis of root development, as in wild-type plants *AGC2-1* showed a high level of expression in this organ. To ascertain the comparable physiological condition of growing roots, we scored the root and root hair phenotypes in a segregating population of seedlings, which was characterised for the status of homozygosity for the T-DNA insertion in the *AGC2-1* gene, using PCR. In general, the mutant roots resembled wild-type roots, and their apical growth rate was also similar to that of the wild type (Table I). However, the length of mature root hairs in the *agc2-1* mutant was only 65% in comparison to the wild type (Figure 6A and B; Table I). Both the root hair phenotype and the hygromycin resistance showed a 3:1 segregation, confirming that the T-DNA insert and the phenotype are genetically linked.

To obtain a tool for further study of AGC2-1 function in root hair development, we have expressed a GFP-AGC2-1 green fluorescent reporter protein in wild-type plants and examined its subcellular localisation. Although these proteins were expressed by the promoter of Cauliflower Mosaic Virus 35S RNA, the pattern of GFP-AGC2-1 expression showed an interesting deviation from a control GFP construct driven by the same promoter. The GFP-AGC2-1 protein displayed an

Figure 5 Expression of AtPDK1 and AGC2-1 mRNA. (**A**) Northern blot with 3′ gene-specific probes of AtPDK1, AGC2-1, cyclin D3 (Riou Khamlichi *et al*, 1999) and the actin depolymerising factor 2 as a loading control (ADF2) (Allwood *et al*, 2002). Samples were chosen to represent various growth and cell division rates: cell suspension in logarithmic growth (CS-log), cell suspension in stationary phase (CS-Stat), roots taken from *Arabidopsis* plants grown in liquid with 3% sugar. (Root SB) or root from Agar plate with 0.5% sugar (Root agar). (**B**) Northern blot of RNA samples from sucrose starvation-induced cell cycle synchronisation experiment. Asynchronous culture (As) and time points of samples taken after re-addition of sugar to the medium are indicated. (**C**) Flow cytometry analysis of the number of cells with G1 and G2 DNA content and the percentages of cells in various cycle stages from selected samples used to prepare the Northern blot in (B).

Table I Root apical growth and root hair length

Line	Root apical growth			Root hair length (μm ± s.e.)	Percentage of wild type
	1 day (mm ± s.e.)	3 day (mm ± s.e.)	7 day (mm ± s.e.)		
Wild type	1.85 ± 0.21	4.82 ± 1.80	11.61 ± 3.62	262 ± 14	—
agc2-1/agc2-1	1.63 ± 0.35	3.63 ± 1.34	13.96 ± 2.99	357 ± 22	65

Root growth was determined by selecting germinated seedlings with roots approximately 15 mm long. A total of 15 seedlings from a wild-type line and 15 from the AGC2-1 knockout were placed on fresh agar plates and displacement of the root tip was measured at 1-, 3- and 7-day intervals. Root hair length was determined by measuring 10 root hairs located 10 mm from the root tip in 18 individual plants from each line.
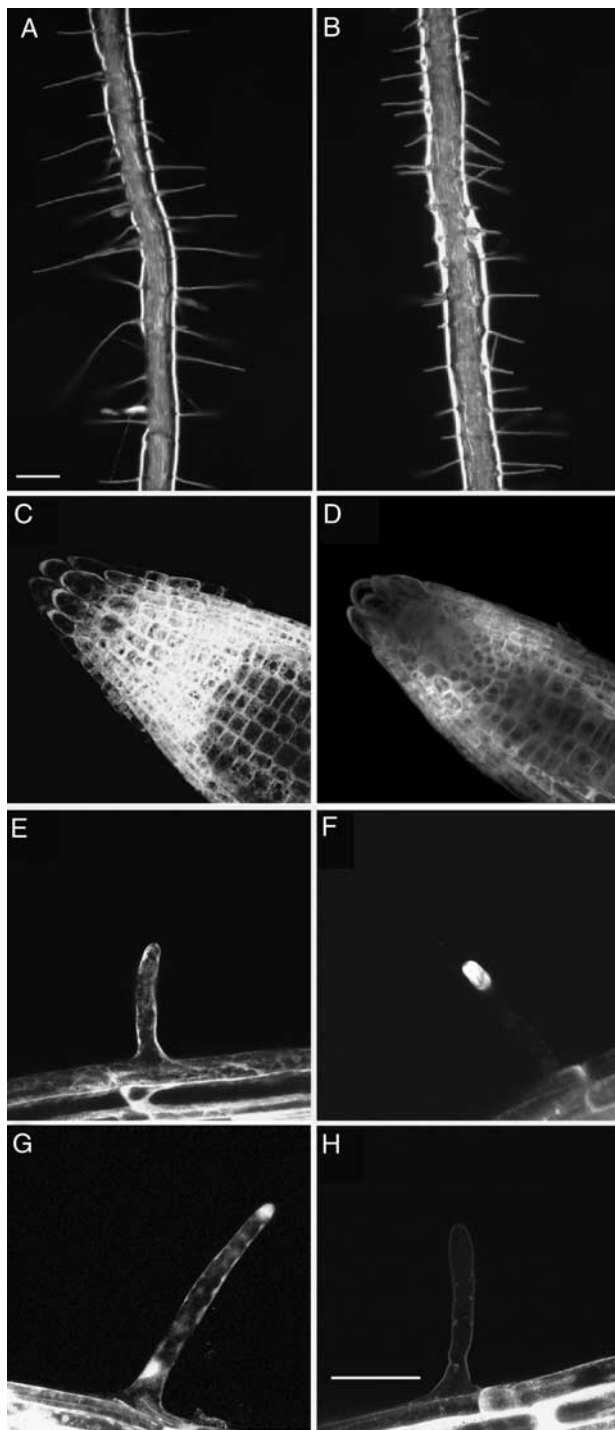
Figure 6 Role of AGC2-1 in root hair growth. (**A**) Root hair phenotype of 7-day-old roots of wild-type and (**B**) the agc2-1/agc2-1 T-DNA insertional knockout mutant, imaged 10 mm from the primary root tip. Scale bar = 100 μM. (**C**) GFP-AGC2-1 localisation in the primary root tip. (**D**) Localisation of GFP control in the primary root tip. (**E**, **F**) GFP-AGC2-1 localisation pattern in immature root hair located 5 mm from the root tip. (**G**) GFP-AGC2-1 localisation pattern 10 mm from the root tip. (**H**) Localisation of GFP control in root hair 10 mm from the root tip. Scale bar = 100 μM.

apparent accumulation in cells of meristematic zones of primary roots (Figure 6C). In root hairs, the localisation of GFP-AGC2-1 reporter protein showed a highly dynamic pattern that characteristically changed during development. In immature root hairs (5 mm from the root tip), GFP-AGC2-1 was localised on the internal surface of root hairs in association with vesicle-like structures (Figure 6E). Later, the GFP-AGC2-1 signal appeared at the root hair tips (Figure 6F) and during root hair growth accumulated around the root hair apex (Figure 6E). In fully elongated hairs, GFP-AGC2-1 also appeared in nuclei positioned at the base of hairs, indicating a developmentally regulated subcellular translocation.

Discussion

PDK1 is central to a number of signalling pathways by activation of downstream protein kinases (Alessi, 2001; Storz and Toker, 2002). We identified a protein kinase in plants, AGC2-1, that is regulated by PDK1.

Two lipids, PI(4,5)P$_2$ and PA, increase the activity of AtPDK1, whereas AGC2-1 activity is only regulated by PA in an AtPDK1-dependent manner. This property of the AtPDK1 enzyme markedly differs from mammalian PDK1, which fails to bind PA, and its activity is not stimulated by PI(4,5)P$_2$ (Alessi *et al*, 1997). It was shown that the presence of sphingosine in *in vitro* assays increases the autophosphorylation of PDK1 as well as the phosphorylation of a number of PDK1 targets, including a novel PDK1 substrate, PAK (King *et al*, 2000). Similarly, a yeast PDK1 homologue, Pkh1, which does not carry the PH domain, has been shown to use sphingosine as a lipid mediator to activate downstream targets involved in the regulation of endocytosis, cell growth, control of mitogen-activated protein kinases and organisation of actin cytoskeleton (Friant *et al*, 2001). We have not been able to demonstrate sphingosine-mediated activation of AtPDK1 or its downstream target AGC2-1. However, it is possible that PA plays a similar role in plants as sphingosine in yeast and animal cells.

The formation of PA has been linked to a variety of plant responses, including biotic or abiotic stresses, dormancy, formation of nodules and hairs on roots (Meijer and Munnik, 2003). The generation of PA is complex in plants, for example, there are 12 members of PLD family in *Arabidopsis*, but it is still largely unknown how these PA-generating enzymes are coupled to downstream signalling pathways. PA has been shown to activate a MAP kinase pathway, but whether this depends on PDK1 or any of the downstream AGC kinases remains to be established (Lee *et al*, 2001). PA production can be elevated by using heterotrimeric G-protein activators, such as mastoporan (Munnik *et al*, 1995). Interestingly, we also found that mastoporan activates AGC2-1 in a PDK1-dependent manner. Involvement of heterotrimeric G-proteins in the regulation of PA-mediated PDK1-AGC2-1 signalling was further corroborated by data from experiments in which an RNAi-G-protein-alpha subunit (RPA1) construct was transfected into *Arabidopsis* protoplasts and resulted in decreased kinase activity of both AtPDK1 and AGC2-1 (RG Anthony *et al*, unpublished data).

Heterotrimeric G-proteins are thought to regulate signalling via G-protein-coupled receptors and modulate plant cell proliferation and auxin-induced lateral root formation (Chen *et al*, 2003; Ullah *et al*, 2003). We have found that two growth-promoting plant hormones, auxin and cytokinin, synergistically activate AGC2-1. However, the response of protein kinase activity to auxin and cytokinin is relatively slow and rises steadily, which suggests that AtPDK1 and

AGC2-1 are not directly controlled by these hormones as primary agonists, but could be important modulators and downstream effectors to hormonal pathways controlling cell elongation, cell division or root differentiation. In *Arabidopsis*, AtPDK1 is expressed in all organs, but *AGC2-1* expression is most abundant in fast-dividing and -growing cells and tissues, similar to other plant protein kinases implicated in growth signalling, the S6K (Zhang *et al*, 1994) and the AtTOR kinase (Menand *et al*, 2002).

In *Arabidopsis*, root hairs offer useful single-cell systems to extend functional studies on the regulatory role of AGC2-1, as our studies indicate that the *agc2-1* knockout mutation results in significant inhibition of root hair growth. Root hair development is known to be controlled by many genetic and environmental factors, including the transcription factors Transparent Testa Glabra (TTG) and GLABRA2 (GL2), light, calcium, pH, free radicals, nutrients, ethylene, auxin and phosphorylation events (Carol and Dolan, 2002). Recent results indicate that GL2 specifically regulates the expression of AtPLD ζ1, which in turn generates PA (Ohashi *et al*, 2003). The localisation pattern of GFP-AtPLD ζ1 and GFP-AGC2-1 is very similar, and thus it will be worth examining whether the established GL2 and AtPLD ζ1 interaction is connected with PA signalling to AGC2-1 via AtPDK1. The association of a phospholipase D isoform with microtubules could provide a mechanism for connecting the cytoskeleton with PA generation, and to regulate signalling pathways locally (Marc *et al*, 1996, Dhonukshe *et al*, 2003). Furthermore, it was shown that inhibition of PLD by *n*-butanol affects root hair elongation (Gardiner *et al*, 2003). Intriguingly, the knockout mutation of the *ire* AGC kinase gene generates a phenotype that is very similar to AGC2-1, that is, the root hairs are about 40% shorter than the wild type (Oyama *et al*, 2002). AGC2-1 and IRE share common structural features, including a conserved activation loop signature and a hydrophobic motif. Based on the kinetics of root hair growth, it was suggested that IRE might associate with microtubules and function as a regulator of tip growth in root hairs.

Auxin promotes root hair elongation (Rahman *et al*, 2002). The auxin response mutants axr1 and aux 1 have short hairs; however, root hair shape and number are unaffected. This correlates with the *agc2-1* knockout that also possessed normal trichoblast cells and root hair shape. AXR1 is localised to the nucleus and is part of a specific protein modification-degradation machinery. The AGC2-1 protein is also nuclear at some developmental stages, and it possesses a putative monopartite nuclear localisation signal.

The results presented in this study provide a framework that describes a novel phospholipid-signalling pathway involving AtPDK1 and its substrate AGC2-1. We show that AGC2-1 is a highly dynamic kinase, regulated by multiple upstream components and implicated in root hair elongation. We do not rule out the possibility that AGC2-1 may be involved in other signalling pathways, potentially with diverse physiological roles.

Materials and methods

Constructs
PCR was used to generate the coding sequence of AtPDK1-1 (accession number At2g01330) using the AtPDK1 clone described previously (Deak *et al*, 1999). AGC2-1, AGC1-1 and AtPDK1-2 were prepared by PCR from an *Arabidopsis* cell suspension library, pACT2CS (Nemeth *et al*, 1998), and cloned into pDONR207 (Invitrogen). C-terminal mutations of the AGC1-1 and AGC2-1 gene (ADFA/ALVA or RGSGC) were produced by incorporating the specific mutations into a 3′ terminal PCR primer followed by PCR of the full-length gene. Internal site-specific mutations in the AGC2-1 gene were carried out using the Gene Editor *in vitro* site-directed mutagenesis system (Promega). Details of the vectors and cloning procedures are provided as supplementary data online.

Protein interaction assays in the yeast two-hybrid system
Saccharomyces cerevisiae strain AH109 was used to screen an *Arabidopsis* pACT2 cDNA library (Nemeth *et al*, 1998) with pAS2-1 bait clones as described previously (Allwood *et al*, 2002). The resulting colonies were initially assessed for reporter gene activity by nutritional selection (growth on medium lacking leucine, histidine and tryptophan, and containing 5 mM of 3-amino-1,2,4-triazole (3-AT)). Yeast colonies were then transferred to nitrocellulose filters on SD-base plates (Clontech) with 2 mM 3-AT. LacZ activity was determined by filter lift analysis including appropriate positive and negative interaction controls.

Cell culture, protoplasting and transfection
Arabidopsis cell suspension culture was maintained as described (Mathur and Koncz, 1998). Protoplast isolation and PEG-mediated transfection were performed according to Meskiene *et al* (2003). For each transfection, a total of 10 μg of plasmid DNA was transfected into 2×10^5 cells and the cells were cultured for 24 to 48 h before harvesting.

GST pull-down assay
Cells were cotransfected with 10 μg of Myc-AtPDK1 plasmid and 10 μg of GST-AGC construct. At 36 h after transfection, the cells were lysed in 0.6 ml of lysis buffer (50 mM Tris–HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (w/v) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 μM microcystin-LR, 0.1% (v/v) β-mercaptoethanol and 1 tablet of protease inhibitor mixture (Sigma) per 50 ml of buffer). The lysates were cleared by centrifugation at 14 000 rpm for 10 min at 4°C, and 0.4 ml of the supernatant was incubated for 2 h at 4°C with 30 μl of glutathione–sepharose. The beads were washed twice in lysis buffer containing 0.5 M NaCl, followed by two further washes in the same buffer. The beads were resuspended in 30 μl of buffer containing 100 mM Tris–HCL pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol and 200 mM dithiothreitol, and subjected to SDS–polyacrylamide gel electrophoresis. The gels were analysed by immunoblotting with anti-HA (clone 3F10; Roche Molecular Biochemicals) or anti-c-Myc (clone 9E10, Santa Cruz Biotechnology) primary antibodies using detection by an enhanced chemiluminescence (ECL) system (Pierce, UK Ltd).

Immunoprecipitation and in vitro kinase assay
In all experiments, samples were prepared in triplicate. Cells were lysed by vortexing frozen samples vigorously for 30 s in 100 μl of extraction buffer and the HA-epitope-tagged protein kinases were immunoprecipitated as described (Meskiene *et al*, 2003). The synthetic peptides were used as substrates: Kemptide, RRASLG, Crosstide, GRPRTSSFAEG, short p70 substrate, AKRRRLSSLRA, long S6 peptide, KEAKEKRQEQIAKRRRLSSLRASTSKSGGSQK, Lkbtide, KKDRWRSMTVVPYLEDKK, PIFtide (REPRILSEEEQEMFRDFDYIA-DEC). Peptides were added to the kinase assay at a final concentration of 30 μM. The PKI inhibitor (YADFIASGRTGRRNAI) was tested at a final concentration of 2.5 μM. A typical reaction contained 30 μM synthetic peptide, 50 mM Tris–HCl, 7.5/0.1% β-mercaptoethanol, 0.1 mM EGTA, 1.0 μM microcysteine, 10 mM magnesium acetate and 100 μM [γ-^{32}P]ATP (1000 cpm/pmol) (NEN Life Science). The kinase reaction was incubated for 30 min at room temperature. A measure of 18 μl of the mix was transferred to p81 discs (Whatman), and washed four times in 0.5% (v/v) phosphoric acid solution. The washed discs were transferred to vials containing 5 ml of scintillation cocktail, and the activity was measured using a scintillation counter. The AGC2-1 rabbit polyclonal antibody was raised against the synthetic peptide CPDDGGDKGTDVNTK. The AtPDK1 rabbit polyclonal antibody was raised against GST-AtPDK1 purified protein extracted from *Escherichia coli*.

The AGC2-1 antibody was affinity purified using the immobilised peptide, with SulfoLink Coupling Gel (Pierce Biotechnology, Rockford, IL). Purified AGC2-1 antibody was then immobilised onto protein A gel with the SeizeX Protein A immunoprecipitation kit (Pierce Biotechnology).

Binding of GST fusion proteins to lipid affinity beads

N-terminal GST-tagged proteins were expressed in *E. coli* using pGEX-KG constructs (Amersham). RPA is the PA-binding region of the animal Raf-1 (residues 390–426) (Rizzo *et al*, 2000), FYVE is a double FYVE domain from mouse Hrs (residues 147–223) (Gillooly *et al*, 2000) and PHOX is the PX domain of human p40^{phox} (NCF-4, residues 1–148) (Kanai *et al*, 2001). A measure of 400 ng of purified protein was used in lipid-binding assays. Proteins were incubated with 6 μl of PI3P beads (9.1% loading) or 3 μl of PA beads (17% loading). Bound protein was eluted from the beads with Laemmli sample buffer, loaded on SDS–PAGE and detected by Western analysis using an anti-GST antibody (Santa Cruz).

Northern analysis

Total RNA and blots were prepared from tissues harvested from *Arabidopsis* var. Columbia ecotype Col O. as described (Allwood *et al*, 2002). Gene-specific 3′ untranslated regions of AtPDK1 and AGC2-1 were generated by PCR. Radiolabelled probes were prepared using ready-to-go DNA-labelling beads [α-^{32}P]dCTP (Amersham).

Sugar starvation and flow cytometric analysis

Arabidopsis cells were synchronised essentially as described (Menges and Murray, 2002), except that 18 g of sorbitol was added to the medium when sucrose was omitted to balance the osmotic pressure. After 24 h, cells were resuspended in the original culture medium, samples were removed and snap-frozen at −80°C every 2 h. Flow cytometry data were obtained using a Partec PAS2 Particle Analysing system (Partec GmbH), using frozen cells without cell wall digestion and chopping as described previously (Bögre *et al*, 1997).

Characterisation of agc2-1 knockout mutant

The agc2-1 mutant was identified in a T-DNA-tagged population (Rios *et al*, 2002) screened with gene- and T-DNA-specific primers. The insertion was placed in the first intron where it totally disrupts the gene. However, Southern analysis showed the presence of other T-DNAs in this line. Plants carrying the insertion were then selfed and crossed to wild-type plants in order to remove the extra T-DNA copies. Root hairs were imaged using a Nikon SMZ1500. Root apical growth and root hair length were determined as described previously (Weigel and Glazebrook, 2002).

Subcellular localisation of GFP-AGC2-1

Roots from homozygote GFP-AGC2-1-expressing lines were mounted in water and examined using a Bio-Rad Radiance 2100 laser confocal microscope. The images were processed with Adobe Photoshop 5 software (Mountain View, CA).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

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