Structural and Functional Insights into the Regulation of Arabidopsis AGC VIIIa Kinases*

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The AGCVIIIa kinases of Arabidopsis are members of the eukaryotic PKA, PKG, and PKC group of regulatory kinases. One AGCVIIIa kinase, PINOID (PID), plays a fundamental role in the asymmetrical localization of membrane proteins during polar auxin transport. The remaining 16 AGCVIIIa genes have not been associated with single mutant phenotypes, suggesting that the corresponding kinases function redundantly. Consistent with this idea, we find that the genes encoding the Arabidopsis AGCVIIIa kinases have spatially distinct, but overlapping, expression domains. Here we show that the majority of Arabidopsis AGCVIIIa kinases are substrates for the 3-phosphoinositide-dependent kinase 1 (PDK1) and that trans-phosphorylation by PDK1 correlates with activation of substrate AGCVIIIa kinases. Mutational analysis of two conserved regulatory domains was used to demonstrate that sequences located outside of the C-terminal PDK1 interaction (PIF) domain and the activation loop are required for functional interactions between PDK1 and its substrates. A subset of GFP-tagged AGCVIIIa kinases expressed in Saccharomyces cerevisiae and tobacco BY-2 cells were preferentially localized to the cytoplasm (AGC1–7), nucleus (WAG1 and KIPK), and the cell periphery (PID). We present evidence that PID insertion domain sequences are sufficient to direct the observed peripheral localization. We find that PID specifically but non-selectively binds to phosphoinositides and phosphatidic acid, suggesting that PID might directly interact with the plasma membrane through protein-lipid interactions. The initial characterization of the AGCVIIIa kinases presented here provides a framework for elucidating the physiological roles of these kinases in planta.

Postembryonic plant development is coordinated through the integration of environmental signals with those derived from intrinsic plant growth regulators. Because the plant cell wall inhibits cell motility and prevents direct cell-to-cell contact, the transmission of information between cells relies largely on the activation of an impressive array of intercellular signaling machinery. The completed sequence of the model organism Arabidopsis has revealed that roughly 10% of the genome is devoted to signaling-related genes (1). Included in this sector are the more than 1,000 annotated genes predicted to encode serine-threonine protein kinases, nearly double the number found in the genomes of representative metazoan organisms (2). One mechanism for the observed expansion of kinases into larger gene families in plants as compared with animals or fungi is the amplification of low copy number genes into larger gene families, which may perform divergent or stage- and tissue-specific functions (3). A major challenge of post-genomic Arabidopsis research will be to understand the extent to which duplicated genes retain ancestral function or are conscripted by other signaling networks to regulate disparate cellular processes.

One example of a gene family that has undergone expansion in plants, is the AGC family of serine-threonine protein kinases. In yeast and mammals, these kinases have been implicated in the regulation of transcription, apoptosis, cell proliferation, insulin signaling, and cytoskeletal remodeling (4–8). Despite the breadth of cellular processes controlled by these proteins, numerous studies suggest that phosphorylation by 3-phosphoinositide-dependent kinase 1 (PDK1), itself an AGC kinase, is an evolutionarily conserved mechanism for regulation of AGC kinase activity (9). In contrast to the detailed information available for their yeast and metazoan counterparts, the signaling processes associated with AGC kinase activity in plants, and the mechanisms by which kinase activity is regulated, remain poorly characterized.

The 39 identified Arabidopsis AGC protein kinases can be phylogenetically grouped into five distinct subfamilies (10). The AGCV1 subfamily includes two S6K homologues that have been shown to respond to regulation by phytohormones (11). The AGCVII class contains eight uncharacterized homologues of the nuclear Dbf2-related kinases (12). The AGC “other” subfamily consists of four genes related to INCOMPLETE ROOT HAIR E LONGATION, which is involved in the modulation of root hair growth (13). The two PDK1 homologues of Arabidopsis cluster together in a separate subfamily. The largest and

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*** The abbreviations used are: PDK1, 3-phosphoinositide-dependent kinase; PID, PINOID; PIF, PDK1-interacting fragment; GST, glutathione S-transferase; His, polyhistidine; GFP, green fluorescent protein; PID-ID, PINOID insertion domain; MBP, myelin basic protein; HRP, horseradish peroxidase.
most diverse kinase class is subfamily VIII, which contains 23 members. The AGCVIII kinases represent a plant-specific subfamily identified by two signature domains; a conserved DFD motif implicated in the coordinate binding of Mg²⁺-ATP, and the presence of a small region of variable sequence inserted between catalytic subdomains VII and VIII (14).

Bögre et al. (10) have further subdivided the AGC VIII subfamily into two classes, VIIIa and VIIIb containing 17 and 6 members, respectively. Three AGC kinases belonging to subgroup VIIIa have been genetically and biochemically characterized in Arabidopsis. The PHOT1 and PHOT2 blue-light photoreceptors contain LOV domains that respond to light and induce a conformational change that activates the kinase domain (15, 16). The third kinase, AGC2–1/OXI1, first identified through its interaction with PDK1, is involved in root hair growth and is activated in response to wounding and pathogen infection (17, 18).

Within the VIIIa subgroup, only PINOID (PID) has been identified genetically, and was subsequently shown to play a fundamental role in the asymmetrical localization of auxin transport proteins at the cell membrane (19–22). A second AGCVIIIa kinase, KIPK, was isolated in a yeast two-hybrid screen for proteins that interact with the kinesin-like motor KCBP (23), although the significance of this interaction is still unclear. More recently, two other kinases of this class, WAG1 and WAG2 were identified in Arabidopsis based on their homology to the light responsive pea gene PsPK3, and have now been shown to redundantly regulate root waving in response to tactile stimulus (24).

With the exception of PID, genetic screens have thus far failed to identify additional mutant phenotypes associated with disruption of AGCVIIIa gene function. Furthermore, while 10 of the 16 genes encoding AGCVIIIa kinases have been identified as carrying insertions within coding regions, plant lines corresponding to the insertion alleles do not have obvious morphological defects. The simplest explanation for these observations is that, like WAG1 and WAG2, the majority of these proteins are likely to exhibit partial or complete functional redundancy. As a first step toward elucidating those processes that are controlled by these kinases, we have initiated the characterization of the AGCVIIIa subfamily in Arabidopsis. Specifically, we have examined gene expression patterns, post-translational activation, and subcellular localization of the corresponding proteins.

**EXPERIMENTAL PROCEDURES**

**AGC Gene Expression Analysis by RT-PCR**—Total RNA was isolated with RNaseasy Plant Mini Kit (Qiagen, Valencia, CA) using 100 mg of plant tissue according to the manufacturer’s protocol. After elution, the RNA was treated for 1 h with RNase-free DNase (Promega, Madison, WI), phenol-chloroform extracted and precipitated. For all RNA samples, the absence of contaminating DNA was confirmed by PCR amplification using the same primers that were used in the RT-PCR analysis.

20 µl of reverse transcription reactions were performed using the RT system (Promega). Total RNA (1 µg) was used as template for the AMV reverse transcriptase in the presence of 0.5 µg of oligo (dT)₁₅ primer according to the manufacturer’s instructions. After first-strand synthesis, the resulting cDNAs were diluted 10-fold, and 10 µl of the diluted solution was used as a PCR template. PCR was carried out in 50-µl reactions using 1 µM of each primer in the presence of 400 µM dNTPs and Taq Polymerase (Promega). Primers for the ubiquitin (UBI) control were added to the reaction tubes prior to the final 24 amplification cycles. All cycles were run at 95 °C for 30 s, 55 °C for 1 min and 72 °C for 40 s. PCR products were separated on a 1.4% agarose gel. Primer sequences used in this study are available upon request.

**Expression and Purification of Recombinant Proteins in Bacteria**—KIPK cDNA was PCR-amplified from clone U22072 (Arabidopsis Biological Resource Center, Ohio State University, Columbus, OH). For all other genes, cDNAs were generated by PCR amplification using total cDNA derived from whole plant or tissue-specific RNA samples, depending on the level and domain of expression of the AGC gene (Fig. 5). Oligonucleotides containing the specific restriction sites used to clone the cDNAs are available upon request. PCR products were sequenced and cloned into the corresponding sites of pGEX4T-1 vector (Amersham Biosciences) to generate N-terminally tagged glutathione S-transferase (GST) fusion proteins. Polyhistidine (His)-tagged PDK1 (At5g04510) was generated as previously described (25). PID-C and WAG1-F mutations were introduced into GST-PID and GST-WAG1 constructs using the QuickChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA). The PIF domain of PID was introduced into WAG1 and WAG1-F by PCR using a 3′-end primer containing PIF domain nucleotides based on the PINOID PIF sequence. PCR products were sequenced and cloned into the pGEX4T-1 vector.

GST and His-tagged constructs were transformed into BL21(DE3) cells. Protein expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside when the A₆₀₀ reached 0.7. Cultures were grown for 18 h at 24 °C, and the bacterial pellets were harvested by centrifugation. Bacterial cells expressing GST-tagged proteins were solubilized in 1× PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.3) containing 1 mg/ml lysozyme and EDTA-free protease inhibitors (Roche Applied Science) and incubated on ice for 30 min. Triton X-100 was added to 1% and the soluble fraction was obtained by centrifugation for 30 min at 8,000 rpm in an SS-34 rotor (Sorvall). The soluble fraction was supplemented with 2 mM dithiothreitol and 120 µl of glutathione-agarose beads (Sigma-Aldrich), and the mixture was agitated at 4 °C for 2 h. The bead-protein complex was washed four times with 1× phosphate-buffered saline.

**GST Pull-down and Immunoblotting**—His-PDK1 bacterial lysate was prepared as described above for the GST-tagged protein except that binding buffer BB (50 mM Tris, pH 6.8, 200 mM NaCl, 0.1% Tween) was used in place of phosphate-buffered saline. GST or GST-tagged AGC proteins bound to agarose beads were incubated with equal volumes of His-PDK1 lysate for 1–2 h at 4 °C with gentle shaking. The beads were washed four times in BB and two times in BB with 300 mM NaCl and 2% glycerol, and the bound proteins were resuspended in Laemmli loading buffer. After separation by SDS-PAGE, the proteins
were transferred to nitrocellulose membrane and the bound PDK1 was visualized by Western blotting with an anti-penta-His primary antibody (Qiagen,) and an HRP-conjugated anti-mouse IgG secondary antibody (Promega). The HRP reaction was developed with ECL Western blotting analysis system (Amersham Biosciences).

**Phosphorylation of AGC Proteins by PDK1—**20 μL of HIS-PDK1 bound to GST-AGC beads was washed once with kinase buffer (KB) (20 mM Tris, pH 7.5, 15 mM MgCl2, 1 mM dithiothreitol), resuspended in 30 μL of KB containing 50 μM ATP and 10 μCi [γ-32P]ATP (3,000 Ci/mmol, MP Biomedicals, Irvine, CA), and incubated for 30 min at 30 °C. Reactions were terminated by adding 10 μL of 4× Laemmli loading buffer, and the samples were separated on 12% polyacrylamide gels (Life Therapeutics, Frenchs Forest, Australia). After electrophoresis, the gels were washed for 1 h in SDS running buffer, stained with Coomassie Brilliant Blue (Bio-Rad), destained with 40% methanol, 10% acetic acid, and then dried for visualization by autoradiography. To determine AGC kinase trans-activity, 8 μg of myelin basic protein (MBP) and 5 μCi of [γ-32P]ATP were added to the phosphorylation reaction after 30 min, and the mixture was incubated at 30 °C for 15 min. Samples containing MBP were separated on 4–20% gradient gel. The band corresponding to MBP was excised from the dried gel, and radioactivity was quantified by liquid scintillation counting.

**Cloning and Expression of GFP-tagged AGC Kinases—**For yeast experiments, the pYES2.0 vector (Invitrogen) was modified by cloning a PCR fragment containing the GFP open reading frame and multicloning site from pEGAD vector (26) into the KpnI and SphI sites to create pYES-GFP. ALSA cDNA was created by introducing mutations into a PID-pGEX4T-1 clone using the QuikChange site-directed mutagenesis kit. cDNAs encoding PID, AGC1–7, ALSA, and KIPK were cloned into pYES-GFP in-frame with GFP using EcoRI-Xhol, and WAG1 into BamHI-SphI restriction sites. For domain swapping between PID and AGC1–7, the PID sequence between the endogenous BglIII and AgeI sites was replaced with a PCR fragment containing the corresponding AGC1–7 sequence resulting in PID-7-PID. 7-PID-7 was created by ligating the PID BglIII-AgeI fragment between 5′ EcoRI-BamHI and 3′ AgeI-Xhol AGC1–7 fragments generated by PCR. The resulting chimeras were subcloned into the EcoRI-Xhol sites of pYES-GFP vector in-frame with GFP. The PID insertion domain (PID-1D), containing amino acids 229–279, was PCR-amplified and subcloned into pYES-GFP vector using EcoRI-Xhol sites.

Constructs were used to transform *Saccharomyces cerevisiae* strain BY2789 (MATα his3-D1 leu2-D0 ura3-D0 lys2-D0) grown onYPD medium using the lithium acetate method as described (27). Cells were grown under conditions that select for plasmid retention in minimal media containing either glucose (SDCAA-URA), or galactose (SGCAA-URA) at 30 °C. For microscopy, logarithmic phase yeast cultures were concentrated to 5 × 10^8 cells/ml by centrifugation at 10,000 × g for 30 s and resuspended in fresh medium.

For expression in tobacco BY-2 cells, wild-type and chimeric GFP fusion constructs were excised from the corresponding pYES-GFP vector with KpnI and Xhol and ligated into the KpnI and Sall sites of the pPZP212 based binary vector CHF3 behind the CaMV 35S promoter. The GFP control plasmid was generated by subcloning the PCR generated KpnI-PstI fragment containing the GFP gene from the pEGAD vector into CHF3. Plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101:pMP90RK by heat shock transformation. Transformed *Agrobacteria* were grown overnight at 28 °C with shaking in YEP broth (yeast extract, 10 g/liter; peptone, 10 g/liter, NaCl, 5 g/liter; pH 7.0) supplemented with 100 μg/ml spectinomycin and 50 μg/ml kanamycin.

3 ml of 3–4-day-old BY-2 tobacco cell suspension cultures were innoculated with 200 μL of *Agrobacteria* (A_600 of 1.5), and the cells were co-incubated with the bacteria in a Petri dish at room temperature in the dark. After 2 days, cells were washed with culture medium 3–4 times, transferred to tubes, and concentrated by centrifugation at 750 × g for 2 min.

Concentrated cells were visualized by pipetting 3 μL onto a microscope slide (Fisher) and applying a coverslip (Fisher). Microscopy was performed on a Zeiss Axiovert 200 M microscope. Images were obtained with a Hamamatsu Orca II camera at ×100 and ×20 objectives for yeast and tobacco cells, respectively.

**Phospholipid Binding—**The binding of PID to biologically active lipids was tested using protein-lipid overlay experiment as described (28). Membranes with bound phospholipids (PIP-strips, P-6001; Echelon Biosciences, Salt Lake City, UT) were blocked in a solution of 3% (w/v) fatty acid-free bovine serum albumin (Sigma; A-7030) in 1× TBS (20 mM Tris, 500 mM NaCl, pH 7.5) for 2 h at room temperature. Polyhistidine-tagged PID protein was purified as previously described (25) and added to the blocking solution. The membranes were incubated overnight at 4 °C. After extensive washes with TBS-T (TBS with 0.1% Tween 20), the membranes were incubated with anti-penta-His primary antibody (Qiagen) followed by an HRP-conjugated anti-mouse IgG secondary antibody (Promega). The HRP reaction was developed with ECL Western blotting analysis system (Amersham). The experiment was performed three times to verify binding results.

**RESULTS**

**Structure of the Arabidopsis AGCVIIIa Kinases—**The AGCVIIIa kinase subfamily is comprised of seventeen predicted proteins that are randomly located throughout the *Arabidopsis* genome (Fig. 1A). The kinases are grouped phylogenetically by both sequence similarity and the presence of several signature domains. In addition to the characteristic DFG to DFD substitution that is shared with the closely related VIIIb group (14), these kinases are uniquely identified by the presence of an insertion domain located between catalytic subdomains VII and VIII (Fig. 1). This domain displays limited sequence similarity between closely related family members, and ranges in size from 48 to 109 amino acids (Fig. 2).

Other sequences common to the majority of the AGC VIIIa kinases are a pair of motifs characteristic of mammalian PDK1 substrates. The first of these sequences is a hydrophobic, C-terminal domain called the PDK1-interacting fragment, or PIF domain. In animals, phosphorylation of the penultimate serine/threonine residue within the consensus PIF sequence, FXFX(S/T)(Y/F), is believed to be required for PDK1 docking (9). 13 of
17 annotated *Arabidopsis* AGCVIIIa kinases contain a C-terminal sequence, F(E/D)XF (Fig. 1B), similar but not identical to the abbreviated PIF variant found in mammalian PKA (29). AtPK7 contains the related sequence IDFF, whereas the remaining three proteins, WAG1, WAG2, and AGC1–12 do not display an obvious C-terminal PIF-like domain (Fig. 1B). In yeast and animals, PDK1 phosphorylates its substrates on the first serine/threonine (P) within a highly conserved activation loop sequence, (S/T)FCGTX(E/D)YXAPE, subsequent to PIF binding (30). A nearly identical sequence found in the plant AGCVIIIa kinases, differs from the consensus PDK1 target site only in the substitution of valine for cysteine at the third residue (Fig. 1B). Two kinases, WAG1 and WAG2, also contain a second amino acid substitution in which the phenylalanine at p + 1 is converted to cysteine (Fig. 1B).

Unlike the conserved catalytic kinase core, which exhibits an average of 81% amino acid identity among all family members, the small insertion domains found in all AGCVIIIa kinases are variable in sequence. However, amino acid alignments reveal that the insertion domains loosely group into five clusters with higher levels of sequence homology (Fig. 2). With the exception of WAG1 and WAG2, the kinases also share a region enriched in basic residues near the C-terminal portion of the domain (Fig. 2). A single kinase, AGC1–10, contains a small acidic domain upstream of the basic region (Fig. 2). Although homology between individual clusters is limited, the insertion domain sequences within a given cluster are conserved between *Arabidopsis* AGCVIIIa kinases and orthologous proteins in both monocot and dicotyledonous plant species (data not shown). Retention of the insertion domains by evolutionarily distant species and conservation of cluster specific sequences among higher plants indicates that these regions are likely to be important for kinase function.

To assess functional differences or similarities between PID and the 16 homologues predicted by the annotated *Arabidopsis* genome, we have cloned cDNAs corresponding to 15 of the genes. We were unable to isolate a cDNA for one homologue, AGC1–8. Digital Northern analysis indicates that transcription of this gene is restricted to root tissues (31), and our inability to detect the transcript suggests that it may be expressed at low levels or in a restricted number of cells within the root.

**In Vitro PDK1 Binding and Phosphorylation of AGCVIIIa Kinases**—In animal systems, phosphorylation of numerous AGC kinases by the upstream regulator PDK1 induces confor-
Regulation of Arabidopsis AGC VIIIa Kinase Family

| Kinase | Sequence | Interaction |
|--------|----------|-------------|
| AGC1–1 | SMA-P | PDK1 binding |
| AGC1–2 | S-A-P | PDK1 binding |
| AGC1–3 | ACCQ-P | PDK1 binding |
| AGC1–4 | ACCQ-P | PDK1 binding |
| AGC1–5 | ACCQ-P | PDK1 binding |
| AGC1–6 | ACCQ-P | PDK1 binding |
| AGC1–7 | ACCQ-P | PDK1 binding |
| AGC1–8 | ACCQ-P | PDK1 binding |
| AGC1–9 | ACCQ-P | PDK1 binding |
| AGC1–10 | ACCQ-P | PDK1 binding |

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Clustering of AGC VIIIa kinases

**Cluster 1**

- **AGC1–1**: SMA-P
- **AGC1–2**: S-A-P
- **AGC1–3**: ACCQ-P
- **AGC1–4**: ACCQ-P
- **AGC1–5**: ACCQ-P
- **AGC1–6**: ACCQ-P
- **AGC1–7**: ACCQ-P
- **AGC1–8**: ACCQ-P
- **AGC1–9**: ACCQ-P
- **AGC1–10**: ACCQ-P

**Cluster 2**

- **AGC1–11**: ACCQ-P
- **AGC1–12**: ACCQ-P
- **AGC1–13**: ACCQ-P
- **AGC1–14**: ACCQ-P
- **AGC1–15**: ACCQ-P

**Cluster 3**

- **AGC1–16**: ACCQ-P
- **AGC1–17**: ACCQ-P
- **AGC1–18**: ACCQ-P
- **AGC1–19**: ACCQ-P
- **AGC1–20**: ACCQ-P

**Cluster 4**

- **AGC1–21**: ACCQ-P
- **AGC1–22**: ACCQ-P
- **AGC1–23**: ACCQ-P
- **AGC1–24**: ACCQ-P
- **AGC1–25**: ACCQ-P

**Cluster 5**

- **AGC1–26**: ACCQ-P
- **AGC1–27**: ACCQ-P
- **AGC1–28**: ACCQ-P
- **AGC1–29**: ACCQ-P
- **AGC1–30**: ACCQ-P

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### FIGURE 2. Sequence alignment of the AGCVIIIa insertion domains.

Sequence alignment of the insertion domains reveals five subclades. Residues in red denote amino acids conserved in more than half of the AGCVIIIa family. Blue, brown, orange, purple, and green-colored amino acids are those common to more than half of the members of individual AGCVIIIa clusters. Stippled bar denotes basic region. The small acidic domain found in AGC1–10 is boxed. Alignments were performed using the Megalign program of Lasergene (DNASTAR).

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Unusually, KIPK, which contains both a C-terminal PIF domain and a consensus signature sequence within the activation loop, has been shown to trigger autophosphorylation in Arabidopsis. This suggests that the PIF domain may be involved in substrate recognition and kinase activation.

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**FIGURE 3.** Pull-down experiments using individual GST-tagged kinases bound to agarose beads and bacterial cell extracts containing His-tagged PDK1 followed by Western blot analysis using anti-His antibody demonstrated that all 15 of the kinases tested interact with PDK1. The observation that WAG1 and WAG2, which lack an obvious PIF domain, bind to PDK1 is consistent with previous experiments in which mutation of the PIF domain did not abolish PDK1 binding and persistent interaction of PDK1 with ADH3 in co-immunoprecipitation experiments following PIF domain mutation.

These data confirm that the PIF domain of the AGC kinases is not absolutely required for PDK1 binding *in vitro*.

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We next examined changes in phosphorylation levels of the AGCVIIIa kinases subsequent to PDK1 co-incubation. As shown in Fig. 3A, the majority of the kinases exhibit a dramatic increase in phosphorylation after incubation with PDK1, indicating that, like its mammalian homologue, *Arabidopsis* PDK1 interacts with multiple AGC kinases. WAG1 and WAG2, which lack both the PIF and the activation loop, are not phosphorylated by PDK1, despite the fact that both proteins bind efficiently to PDK1 in pull-down assays (Fig. 3B). Interestingly, phosphorylation of AGC1–12, which also lacks the C-terminal PIF domain, was stimulated by co-incubation with PDK1 (Fig. 3A). Closer inspection of the AGC1–12 amino acid sequence revealed a consensus PIF sequence (FELF)–100 amino acids upstream of the protein terminus (Fig. 1). It is possible that this site functions as a cryptic PIF domain, and may explain the increase in phosphorylation in response to PDK1.
increased phosphorylation after co-incubation with PDK1, and one, WAG1, which is not phosphorylated by PDK1, to phosphorylate the exogenous substrate MBP. As shown in Fig. 3C, phosphorylation of AGC1–4, AGC1–6, and AGC1–12, by PDK1 resulted in 2–9-fold increase in transphosphorylation. By contrast, prior incubation of WAG1 with PDK1 had no effect on its activity. Collectively, our data argue that *Arabidopsis* PDK1 is likely to be an important, but not universal, regulator of AGCVIIIa kinase activity.

**Functional Analysis of PIF Domain and Activation Loop Sequences**—Two peptide motifs, the PIF domain and an activation loop containing phenylalanine at the p + 1 position (Fig. 1), are present in all AGCVIIIa kinases that are activated by PDK1. To assess the contribution of these motifs to PDK1 interaction and AGC kinase activity, we introduced both motifs, alone and in combination (Fig. 4A), into the WAG1 kinase, which is not an *in vitro* substrate for PDK1.

We first asked whether the addition of a PIF domain was sufficient to convert WAG1 into a substrate for PDK1. Somewhat surprisingly, the addition of a C-terminal PIF domain to WAG1 (WAG1-PIF and WAG1-FPIF)-stimulated kinase autophosphorylation even in the absence of PDK1 (Fig. 4A, compare lane 1 with lanes 5 and 7). Co-incubation of either construct with PDK1 resulted in a decrease in protein phosphorylation, although levels remained elevated as compared with the wild-type protein (Fig. 4A). The observed decrease in phosphorylation in response to PDK1 co-incubation was reflected in a concomitant decrease in the activity of WAG1-FPIF toward MBP substrate (Fig. 4B). Thus, while addition of a PIF domain resulted in an overall increase in autophosphorylation, the...
mechanism of PIF-mediated activation appears to be largely independent of PDK1 function.

We next asked whether conversion of the activation loop cysteine to phenylalanine, alone or in tandem with the PIF domain, resulted in increased phosphorylation of the modified protein by PDK1. Substituted WAG1 derivatives showed increased phosphorylation irrespective of the presence of PDK1 or the PIF domain (Fig. 4A, compare lanes 1 and 2 with lanes 3 and 4, and lanes 5 and 6 with lanes 7 and 8). Reciprocal experiments in which the conserved activation loop phenylalanine of PID was replaced by cysteine (PID-C) resulted in a small but reproducible decrease in phosphorylation as compared with wild type, both before and after PDK1 stimulation (Fig. 4C). These data suggest that while the conserved phenylalanine enhances kinase autophosphorylation, it is unlikely to function in PDK1 phosphorylation site recognition. Collectively, our data indicate that neither the PIF domain nor the activation loop phenylalanine alone or together are sufficient to confer PDK1 substrate identity.

The AGCVIIIa Genes Are Differentially Expressed—We used semi-quantitative RT-PCR analysis of RNA extracted from seven adult tissue fractions and 10-day old seedlings to examine the expression of AGCVIIIa kinases. Consistent with our inability to clone the AGC1–8 cDNA, we were unable to reproducibly detect a transcript for the hypothetical gene in any of the tissues tested (data not shown). The other sixteen kinases showed a spectrum of expression patterns (Fig. 5). With the exception of AGC1–4 and AGC1–7, all of the kinases appear to be expressed in young seedlings. In adult tissues, AGC1–1 and AGC1–2, which group tightly in both whole protein and insertion sequence alignments, were ubiquitously expressed in all tissues examined. The closely related gene AtPK7 was expressed in all tissues except rosette and cauline leaves. In contrast, AtPK5 was expressed only in certain aerial tissues including the inflorescence, flowers, and siliques. Another closely related gene pair, KIPK and AGC1–9, also showed a nearly identical expression pattern, with both genes expressed in all tissues with the possible exception of rosette leaves, and having highest expression in the inflorescence. AGC1–3 and AGC1–4, which are also phylogenetically paired, had overlapping expression domains. AGC1–3 was expressed in flowers, inflorescences, roots and siliques, while AGC1–4 expression was limited to flowers, inflorescences and roots. The subclade containing AGC1–5, AGC1–6, and AGC1–7 displayed a highly divergent expression pattern. Whereas AGC1–5 was expressed in all tissues, AGC1–7 expression appeared to be restricted to flowers and roots, whereas in adult tissues AGC1–6 expression was observed only faintly in the root. WAG1 was expressed in all adult tissues, whereas WAG2 expression was excluded from leaves, stems, and siliques. In adult tissues, AGC1–12 expression was restricted to inflorescences and to a lesser extent to flowers. AGC1–10 expression was not observed in any of the adult tissues examined. Consistent with its loss-of-function phenotype, PID expression was found primarily in inflorescences and flowers, with only trace levels detectable in other adult tissues.

Subcellular Localization of Arabidopsis AGCVIIIa Kinases—To determine whether AGCVIIIa kinases are differential distributed within the cell, we examined the localization of full-length AGC1–7, WAG1, KIPK, and PID fused to GFP. Because exogenous expression of regulatory proteins in plants often results in low protein yield, cell and tissue lethal-
ity, and the co-suppression of native and exogenous genes, we first addressed subcellular kinase localization by expressing AGCVIIIa-GFP fusions in *S. cerevisiae*.

Visualization of GFP in yeast following induction of transgene expression revealed that GFP-AGC1–7 showed cytoplasmic localization indistinguishable from that observed for the GFP-only control (Fig. 6, A and E). By contrast, GFP-KIPK and GFP-WAG1 expression were predominantly nuclear (Fig. 6, C and D). The apparent nuclear localization of GFP-WAG1 in yeast is similar to that observed after transient expression of GFP-tagged WAG1 and WAG2 in onion epidermal cells.5 (Significantly, the GFP-PID fusion protein was preferentially localized to the cell periphery (Fig. 6F) in a manner similar to that observed following *in planta* expression of PID under the control of the root hair specific AtEXPA7 promoter (33). Additional punctate PID expression was observed within cytoplasmic structures, suggesting that PID may also accumulate in one or more endomembrane compartments (Fig. 6F). Alternatively, the punctate expression may result from artifactual protein aggregation.

To confirm that the observed subcellular localization reflected biologically significant protein interactions as opposed to nonspecific binding with yeast cellular components, we transformed tobacco BY-2 cell cultures with GFP-PID and GFP-AGC1–7. The resulting localization patterns were similar to those observed in yeast transformants, confirming that AGC1–7 and PID are preferentially localized to the cytoplasm and cell periphery, respectively (Fig. 6, G and H).

We next asked whether the observed localization of PID relied on prior activation by PDK1. A mutant GFP fusion construct, GFP-ALSA was expressed in yeast. In this mutant the two activation loop serines required for PDK1 activation of PID (25) were replaced by alanine. The resulting protein, which has only low levels of basal activity, exhibited a localization pattern identical to that of the wild-type protein (Fig. 6B), indicating that phosphorylation by PDK1 is not required for proper protein localization.

### FIGURE 5. Expression of the Arabidopsis AGCVIIIa gene family in seedlings and adult tissues
Semi-quantitative RT-PCR using gene-specific primers shows the organ-specific transcript distribution for the AGCVIIIa kinases in seedlings and in seven adult organs. The data are representative of at least two independent experiments.

### FIGURE 6. Subcellular localization of GFP-tagged AGC kinases
Yeast (A–F) and tobacco (G and H) cells expressing GFP-tagged AGC kinases were visualized by differential interference contrast (DIC, *left panels*) and epifluorescence (*right panels*) microscopy. C and D, GFP-KIPK and GFP-WAG1 proteins are targeted to the nuclear region and show low level cytoplasmic accumulation. A, E, and G, GFP and GFP-AGC1–7 accumulate in the cytoplasm. B, F, and H, GFP-ALSA and GFP-PID are localized to the cell periphery.

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5 J. Watson, personal communication.
Regulation of Arabidopsis AGC VIIIa Kinase Family

The PIF domain of the Arabidopsis AGCVIIIa kinases closely resembles the compact PIF domain found in the catalytic subunit of mammalian PKA. Yeast two-hybrid analysis, peptide phosphorylation assays and co-crystalization of PKA and PDK1 have shown that the interaction of these proteins is achieved in part through sequestration of the PKA PIF domain within a hydrophobic pocket located in the anterior lobe of PDK1 (29, 35). Protein contacts established through the interaction of the PIF domain with the hydrophobic PIF pocket have been inferred to function in substrate recognition via protein docking and in the facilitation of phosphotransfer through substrate mediated stabilization of active PDK1 conformation (29). Similar hydrophobic pockets have now been identified in multiple AGC kinases and crystal structure analysis indicates that subsequent to PDK1 activation, the PIF domains of substrate kinases associate intramolecularly with their own PIF pockets to lock the protein in an active conformation.

Our *in vitro* binding data show that all of the AGCVIIIa kinases tested, including WAG1 and WAG2, which lack the PIF motif, were able to interact with PDK1. These data are in agreement with previous reports in which substitution of conserved residues within the PIF domains of PID and Adi3 resulted in dramatic decreases in protein phosphorylation, whereas having only modest effects on protein binding (25, 32). However, Anthony et al. (17) used yeast two-hybrid analysis to demonstrate that an intact PIF domain is essential for the interaction of AGC1–1 with PDK1 in *vivo*. Together these data indicate that while sequences outside of the PIF domain play a role in protein-protein interactions *in vitro* and may contribute to protein affinities *in vivo*, the presence of a PIF domain appears to be a critical factor for functional interactions between PDK1 and plant AGCVIIIa kinases.

Several lines of evidence also indicate that sequences outside of the PIF domain contribute to substrate specificity. First, KIPK, which contains a consensus PIF domain, is not phosphorylated by PDK1 *in vitro*. Second, while the addition of a PIF domain to WAG1 resulted in an overall increase in kinase auto-phosphorylation, co-incubation with PDK1 antagonized kinase activation. These points strongly suggest that the PIF domain alone is not sufficient to mediate PDK1 interaction. One explanation for the PDK1 independent activation of WAG1-PIF is that it may retain an ancestral PIF pocket that recognizes and binds the exogenous PIF sequences. In this model, co-incubation with PDK1 would result in decreased WAG1-PIF activation through competition for PIF binding. Finally, AGC1–1 purified from bacteria (our data) but not from plants (17) serves as an *in vitro* substrate for PDK1, indicating that additional post-translational modifications are likely to impact the ability of PDK1 to regulate AGCVIIIa activity in plants.

The lack of a PIF domain in WAG1 and WAG2 correlates with the conversion of a conserved phenylalanine in the activation loop to cysteine. We asked whether this amino acid (34). By contrast, the structurally distinct AGCVIIIa kinases represent a plant-specific kinase subfamily whose functional characterization has been complicated by the lack of single mutant phenotypes. In this study, we have examined the regulation of the Arabidopsis AGCVIIIa kinase subfamily as a first step in understanding protein function.

**DISCUSSION**

The AGC kinases constitute a highly conserved class of regulatory proteins that are present in all eukaryotic lineages. Orthologues of many of the plant AGC classes, including PKC, Dbf2, SGK, S6K, and PDK1 appear to be present in all eukaryotes. Furthermore, the ability of human PDK1 and SGK to rescue the lethality associated with the corresponding yeast mutants indicates that the signaling processes regulated by these kinases are both ancient and evolutionarily conserved peripheral localization pattern identical to that of intact GFP-PID (Fig. 7, B and D).

To determine whether the insertion domain was sufficient to direct protein distribution within the cell, we transformed yeast with an insertion domain construct fused to GFP (PID-ID) in yeast. Fig. 7, protein-lipid overlay showing binding of PID to phosphorylated inositides and PA. LPA, lysophosphatidic acid; LPC, lyso-phosphocholine; PI, PtdIns; PI3P, PtdIns(3)P; PI4P, PtdIns(4)P; PI5P, PtdIns(5)P; PE, phosphatidylethanolamine; PC, phosphatidylcholine; STP, sphingosine 1-phosphate; PIP3, PtdIns(3,4)P2; PIP2, PtdIns(3,5)P2; PI(4,5)P2, PtdIns(4,5)P2; PI(3,4)P2, PtdIns(3,4)P2; PI(3,5)P2, PtdIns(3,5)P2; PA, phosphatidic acid; PS, phosphatidylserine.

Peripheral localization pattern identical to that of intact GFP-PID (Fig. 7, B and D).

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affected PDK1 binding by introducing a corresponding substitution into the wild-type PID protein. Mutation of this residue did not affect PDK1-dependent PID activation. Furthermore, substitution of phenylalanine for the cysteine in WAG1 or WAG-PIF had no effect on PDK1 interaction. Given that the remaining Arabidopsis AGCVIIIa kinases are absolutely conserved in this region, it seems likely that sequences that modulate PDK1 substrate affinity are likely to reside within the N- or C-terminal non-conserved regions, or within the kinase domain itself.

Because the majority of AGCVIIIa kinases appear to interact similarly with PDK1, it seems likely that mechanisms in addition to kinase activation must determine substrate specificity. To this end, we investigated transcript distribution as a source of functional specificity between closely related kinases. Our finding that numerous AGCVIIIa genes exhibit either stage-specific (AGC1-6 and AGC1-10) expression, or expression confined to a limited number of organs (PID, AGC1-3, AGC1-4, AGC1-7, AGC1-12, and ATPK5), indicates that, while these loci encode highly conserved catalytic domains, the corresponding regulatory sequences appear to have undergone extensive diversification. Notably, multiple AGCVIIIa genes were found to be expressed in each organ type tested. Although we cannot exclude the possibility that expression within a given organ is restricted to a specific cell or tissue type, the overlapping expression domains may in large part explain the lack of loss-of-function phenotypes associated with these genes.

An effective mechanism to further restrict the functional specificity of kinases, such as PDK1, with multiple downstream targets, is through differential localization of substrates to distinct subcellular regions. The preferential localization of three Arabidopsis AGCVIIIa kinases to perinuclear regions (KIPK and WAG1) and the cell periphery (PID) in yeast indicates that individual kinases are targeted to discrete locations through interaction with distinct cellular components. It has previously been proposed that interaction with PDK1 might recruit PID to the plasma membrane (25). However, several observations from the present study call this model into question. First, nearly all of the Arabidopsis AGCVIIIa kinases interact with PDK1 in vitro, but thus far only PID has been demonstrated to exhibit affinity for the plasma membrane or plasma membrane-associated proteins. Second, the mutation of a pair of activation loop serines previously shown to be phosphorylation targets for PDK1 did not affect protein localization. Finally, the affinity of PID for phosphoinositides raises the possibility that membrane localization may be an intrinsic property of the protein.

The above observations suggest that activation and localization of PID are separable functions. This result is in contrast to the findings of Lee and Cho (33) who previously reported that expression of a kinase-dead PID protein variant partially lost the peripheral localization characteristic of wild-type PID when expressed in Arabidopsis root hair cells. It is possible that in the context of the full-length protein, the substitution of Asp205, which maps 23 amino acids upstream of the start of the insertion domain, with alanine might result in a conformational change that prevents efficient interaction between the insertion domain and its peripherally localized binding partner.

Changes in protein localization associated with the reciprocal exchange of insertion domains between PID and AGC1–7 and the ability of the isolated insertion domain to properly localize provide compelling evidence that this protein domain is sufficient to target PID to the cell periphery. Among the metazoan AGC kinases, members of the NDR subfamily, which mediate polarized cell growth and cytokinesis, contain a 30–60 amino acid insertion at a position corresponding to that occupied by the insertion domain of plant AGCVIIIa kinases (12). This domain harbors a non-consensus localization signal that targets NDR kinases to the nucleus (37). The similarity in domain size and function may imply that protein folding optimally positions sequences in this region for interaction with partner proteins or other structural components of the cell to mediate localization.

The close agreement between yeast and tobacco expression data indicates that plant AGCVIIIa kinases differentially distribute to discrete subcellular regions. The observed protein distributions are likely to reflect the differential affinity of plant kinases for distinct binding partners. It will be important to determine whether the insertion domains of other AGC kinases are, like that of PID, the primary determinant of sub-cellular localization. The ability to correlate similarity in insertion domain structure with distinct subcellular localization patterns will be an invaluable tool in the identification of candidate pairs or groups of functionally redundant proteins. Ultimately, the identification of both binding partners and substrates will need to be unambiguously established to assign individual AGCVIIIa kinases to specific signaling pathways, a first step in understanding the biological roles of these regulatory proteins in plant development.

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