AtDGK2, a Novel Diacylglycerol Kinase from Arabidopsis thaliana, Phosphorylates 1-Stearoyl-2-arachidonoyl-sn-glycerol and 1,2-Dioleoyl-sn-glycerol and Exhibits Cold-inducible Gene Expression*

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Received for publication, November 6, 2003, and in revised form, December 4, 2003
Published, JBC Papers in Press, December 9, 2003, DOI 10.1074/jbc.M312187200

Diacylglycerol kinase (DGK) phosphorylates diacylglycerol (DAG) to generate phosphatidic acid (PA). Both DAG and PA are implicated in signal transduction pathways. DGKs have been widely studied in animals, but their analysis in plants is fragmentary. Here, we report the cloning and biochemical characterization of AtDGK2, encoding DGK from Arabidopsis thaliana. AtDGK2 has a predicted molecular mass of 79.4 kDa and, like AtDGK1 previously reported, harbors two copies of a phorbol ester/DAG-binding domain in its N-terminal region. AtDGK2 belongs to a family of seven DGK genes in A. thaliana. AtDGK3 to AtDGK7 encode ~55-kDa DGKs that lack a typical phorbol ester/DAG-binding domain. Phylogenetically, plant DGKs fall into three clusters. Members of all three clusters are widely expressed in vascular plants. Recombinant AtDGK2 was expressed in Escherichia coli and biochemically characterized. The enzyme phosphorylated 1,2-dioleoyl-sn-glycerol to yield PA, exhibiting Michaelis-Menten type kinetics. Estimated $K_{m}$ and $V_{max}$ values were 125 $\mu$M for DAG and 0.25 pmol of PA min$^{-1}$ $\mu$g$^{-1}$, respectively. The enzyme was maximally active at pH 7.2. Its activity was Mg$^{2+}$-dependent and affected by the presence of detergents, salts, and the DGK inhibitor R59022, but not by Ca$^{2+}$. AtDGK2 exhibited substrate preference for unsaturated DAG analogues (i.e. 1-stearoyl-2-arachidonoyl-sn-glycerol and 1,2-dioleoyl-sn-glycerol). The AtDGK2 gene is expressed in various tissues of the Arabidopsis plant, including leaves, roots, and flowers, as shown by Northern blot analysis and promoter-reporter gene fusions. We found that AtDGK2 is induced by exposure to low temperature (4 °C), pointing to a role in cold signal transduction.

* This work was supported in part by German Academic Exchange Service (DAAD) Fellowship A/00/11636 (to F. C. G.-M.) and by DAAD within the framework of the Project Based Personnel Exchange Programme (ARC-XVI-I). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** Supported by British Council British German Academic Research Program Grant 1188.

† Supported by a Ph.D. fellowship from the Egyptian Ministry for Higher Education.

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Eukaryotic diacylglycerol kinases (DGKs); ATP,1,2-diacylglycerol 3-phosphotransferases; EC 2.7.1.107) are a widespread family of enzymes, well conserved among most multicellular organisms. These enzymes phosphorylate diacylglycerol (DAG) to produce phosphatidic acid (PA). DAG is released by the catalytic activity of phosphoinositide-specific phospholipase C (PLC) from phosphatidylinositol-4,5-bisphosphate, and inositol-1,4,5-trisphosphate is generated by the same reaction. Both DAG and PA are second messengers and play a central role in the biosynthesis of major phospholipids. In animal cells, DAG allosterically activates protein kinase C (PKC) (1, 2) and regulates the activity of other proteins involved in carcinogenesis and metastasis as well as in cell growth, development, survival, and apoptosis (1, 3–6). In plant cells, DAG has also been demonstrated to induce both ion pumping in patch-clamped guard cell protoplasts and opening of intact stomata (7). PA also signaling functions; in animal cells, it elicits several biological responses, including cytoskeletal organization by inducing actin polymerization and stress fiber formation (2) and regulates the activity of diverse enzymes (8–12). In plants, PA accumulates in response to various stimuli including cold, wounding, or pathogen attack (13) and also modulates the activity of several enzymes (8). DGK activity is likely to be tightly controlled to regulate DAG and PA levels coordinately, allowing the cell to perform its proper physiological functions. In addition to the PLC/DGK pathway, PA can also be generated by the activity of phospholipase D.

In mammals, nine different DGK isozymes have been reported so far (14). No DGK gene has yet been discovered in Saccharomyces cerevisiae. It was therefore suggested that signaling-related DGKs may control physiological functions unique to multicellular organisms (15). In plants, functional analysis of DGK genes is largely absent. Katagiri et al. (16) reported the molecular cloning of a cDNA (AtDGK1) putatively...
encoding a DGK from *Arabidopsis thaliana*. Unfortunately, AtDGK1 was not enzymatically active when expressed in either *Escherichia coli* or mammalian (COS-7) cells, leaving the question open whether AtDGK1 indeed represents a DGK. Northern blot experiments indicated that AtDGK1 is expressed in root, leaf, and shoot tissue but not in flowers or silique (16). In tomatoes, Snedden and Blumwald (17) isolated a cDNA, designated LeCBDGK, encoding a calmodulin-binding DGK isoform. Calmodulin (CaM) is a Ca\(^{2+}\)-binding regulatory protein widely distributed in plants and animals. In LeCBDGK, the CaM-binding domain was located close to the C terminus of the protein. A second cDNA, LeDGK1, was isolated and found to be identical to LeCBDGK, except that it lacked the codons encoding the CaM-binding domain. The two transcripts are generated by alternative splicing from the same gene. Both DGK isoforms were found to be catalytically active when expressed in *E. coli*. An effect of CaM on the catalytic activity of recombinant LeCBDGK was not discovered. However, in the presence of Ca\(^{2+}\), LeCBDGK associated with membrane cell fractions in vitro. This association was disrupted by the addition of CaM antagonists, suggesting that Ca\(^{2+}\) plays a role in recruiting the enzyme from soluble to membrane cell fractions (17).

The physiological role DGKs play in plants is not precisely known at the present stage. Recently, Ruelland et al. (18) investigated the response of *Arabidopsis* suspension cells to cold treatment. These researchers discovered that the content of PA rapidly, within minutes, increases in response to a temperature drop. This elevation was largely due to a simultaneous activation of the PLC/DGK pathway, although a minor fraction (20\%) of the PA was generated by phospholipase D activity. Inhibitors affecting either PLC or DGK reduced PA accumulation (18).

Genes for enzymes of the plant phosphoinositide pathway have been discovered in recent years (reviewed in Ref. 19), and cold induction of *AtPLC1* (encoding phosphoinositide-specific phospholipase C) (20) has been reported.

This paper reports the molecular characterization of *AtDGK2* from *Arabidopsis thaliana*. We demonstrate that recombinant AtDGK2 enzyme raised in *E. coli* is catalytically active, generating PA from DAG. AtDGK2 is transcribed in a number of tissues, including roots, leaves, and flowers. Its transcriptional activity is promoted by cold treatment, indicating that AtDGK2 may participate in cold signaling. Part of this work has previously been published as a scientific meeting report (21).

**EXPERIMENTAL PROCEDURES**

**General**—Standard molecular-biological techniques were performed as described (22). Restriction enzymes were purchased from Roche Applied Science (Mannheim, Germany) and New England Biolabs (Frankfurt am Main, Germany). Oligonucleotides were obtained from TikiMolbiol (Berlin, Germany). DNA sequencing was performed by AGOWA (Berlin, Main, Germany). Oligonucleotides were obtained from TibMolbiol (Berlin, Germany). DNA and cDNA were sequenced by the NCBI Gene Expression Omnibus data repository was accessed via the World Wide Web at www.ncbi.nlm.nih.gov/).

Cloning of *AtDGK2 cDNA*—Amplification by PCR was carried out using the Advantage-HF2 PCR kit (Clontech, Heidelberg, Germany) according to the manufacturer’s protocol. *A. thaliana* (L.) Heynh. C24 leaf cDNA was used as template. Primer sequences were as follows: primer O-DGKFor (5′-primer), 5′-ATGATGAGCGCTTCCTTTACCC-3′; primer O-DGKRev (3′-primer), 5′-CTACGAGAGATGGACAGCCT-TTATTC-3′; primer O-DGK1Rev (3′-primer), 5′-CTACGAGAGATGGACAGCCT-TTATTC-3′; primer O-DGK1Rev (3′-primer), 5′-CTACGAGAGATGGACAGCCT-TTATTC-3′; primer O-DGK1Rev (3′-primer), 5′-CTACGAGAGATGGACAGCCT-TTATTC-3′. Reaction details were as follows: 95 °C for 50 s; 35 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min; 72 °C for 10 min. PCR products were analyzed by agarose gel electrophoresis. Individual fragments were purified using the QiAquick PCR purification kit (Qiagen, Hilden, Germany) and subcloned into pCRII (Invitrogen). The entire *AtDGK2* cDNA, present in plasmid pCR2.1-dgk2, had a length of 2139 bp (deposited under GenBank accession number AY380783).

**Northern Blot Analysis**—Total RNA isolated from 10-week-old *A. thaliana* C24 plants raised on soil was used for Northern blot analysis. Total RNA (45 μg of RNA) was loaded per lane on 1.5% agarose gels and separated by electrophoresis under denaturing conditions. RNA was blotted onto Hybond-N nylon membrane (Amersham Biosciences) and cross-linked by UV illumination. Prehybridization was done for 2 h at 62 °C in sodium phosphate buffer (0.25 M, pH 7.2) containing 7% SDS, 1% bovine serum albumin, and 1× EMDTA. The AtDGK2 cDNA was radioactively labeled with [α-32P]dCTP (Hartmann Analytic, Braunschweig, Germany) using the Rediprime II DNA labeling kit (Amersham Biosciences). Hybridization was performed overnight at 65 °C in sodium phosphate buffer. The membranes were washed twice for 15 min at 65 °C (first wash: 5× SSC, 0.5% SDS; second wash: 1× SSC, 0.5% SDS). Membranes were exposed to x-ray films (Kodak X-Omat AR) at −80 °C for 1 day.

**Promoter-GUS Fusions**—A 1.2-kb 5′ genomic fragment upstream of the ATG start codon of the *AtDGK2* gene was amplified by PCR using primers PromDGKFor (5′-CCCCAGGCCTATCTGACTAATATGTT-AAAATCGT-3′); added HindIII site underlined) and PromDGKRev (5′- CATTGCATGCTTGTAATCATATACTGT-CC-3′; added Ncol site underlined). A. thaliana (L.) Heynh. C24 genomic DNA served as template. Cycling conditions were as follows: 95 °C for 50 s; 35 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 2 min; 72 °C for 10 min. The amplified promoter fragment was inserted into plasmid pCR2.1 (Invitrogen). Subsequently, the promoter was fused via HindIII/NcoI to the β-glucuronidase (GUS) reporter gene in pCAMBIA-1303 (available on the World Wide Web at www.cambia.org.au/main/r_et_quick-pick.htm). The same promoter fragment was also tested in plants employing binary vector pGPTV-HPT! (available on the World Wide Web at www.biotech.unl.edu/transgenic/vectors.html).

The final constructs were transformed into Agrobacterium tumeefaciens strain GV3101 (pMP96), and transgenic *Arabidopsis* plants were obtained using the floral dip method (23). Transgenic plants were selected on sterile medium containing hygromycin.

GUS activity was determined in vacuum-infiltrated plants or tissues using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) as substrate (24). Infiltrated plants were incubated in GUS staining solution overnight at 37 °C. GUS staining patterns were viewed with a Leica MZ 12.5 stereo microscope (Leica Microsystems, Wetzlar, Germany). Tissue sections (15 μm) were cut with a Leica RM 2155 microtome (Leica Instruments, Nussloch, Germany) and viewed under an Olympus AX70 TRR-AF1 microscope.

**Low Temperature, ABA, and Drought Response Assay**—Seeds of wild-type *A. thaliana* ecotype C24 and transgenic *Arabidopsis* plants transformed with the *promAtDGK2*::GUS fusion construct were sown in a 1:1 (v/v) mixture of soil (GS90; Gebr. Patzer, Sinntal Jossa, Germany) and vermiculite (Deutsche Vermiculite GmbH, Nieder-Erlenbach, Germany) and cultured under controlled environmental conditions in growth chambers with an irradiance of 150 μmol at a photoperiod given above. After treatment, plants were transferred to a growth chamber under the same conditions except that the photoperiod was reduced to 8 h. Twenty-eight days after germination, plants were kept under 250 μmol, 20 °C, 80/50% relative humidity (day/night). Low temperature treatments were carried out as follows: (i) confining plants in different ages to growth chambers set at 4 °C for different periods of time under the light and photoperiodic conditions given above. After treatment, plants were either frozen in liquid nitrogen for RNA isolation (wild-type plants) or were subjected to GUS analysis (transgenic plants). For ABA (Sigma) treatments, mixed isolomers of the phytohormone were dissolved in 10 ml of 10 M LiCl. Plants were sprayed with a final concentration of 10 M ABA and harvested at different time points. Control treatments were given by spraying plants with water containing the same final concentration of NaOH. Drought stress was applied to whole transgenic plants by omitting watering. Plants were sampled after 2 and 5 days.
Expression of Recombinant AtDGK2 in E. coli—The coding region of the AtDGK2 cDNA, flanked by EcoRI sites, was transferred from plasmid pCR2.1-dgk2 to plasmid pET43c (Novagen, Darmstadt, Germany), yielding plasmid pET43c-dgk2. The pET43c vector allows the generation of fusion protein containing a NusA tag (facilitating the solubility of membrane-associated proteins), and a His tag (for detection and purification of target proteins; see Novagen, on the World Wide Web at www.novagen.com). Plasmid pET43c-dgk2 was transformed into E. coli BL21 (DE3) (Novagen). Cells were grown at 37 °C. At an A 600 of 0.2–0.3, expression of NusA-His-AtDGK2 fusion protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (1 mM final concentration). Cells were harvested 4 h later by centrifugation; resuspended in lysis buffer containing 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10 mM imidazole, and Complete Mini Protease Inhibitor Mixture (Roche Applied Science); and lysed by sonification. Recombinant AtDGK2 was purified using Ni 2+ -nitrilotriacetic acid-agarose according to the manufacturer’s (Qiagen) protocol. All homogenates were frozen and stored at −80 °C until assayed. Protein concentration was determined according to Bradford (25), using bovine serum albumin as a standard.

Enzyme Assays—Diacylglycerol kinase activity was determined by measuring the incorporation of [γ-32P]ATP into phosphatidic acid at 25 °C. The standard assay contained in a total volume of 250 μl: 40 mM Bis-Tris (pH 7.5), 5 mM MgCl2, 0.1 mM EDTA, 1 mM spermine, 0.5 mM dithiothreitol, 1 mM sodium deoxycholate, 0.02% Triton X-100, 250 μM cardiolipin, 500 μM 1,2-DOG, 1 mM ATP (containing ~5 μCi of labeled ATP), and approximately 1 μg of the purified enzyme fraction. The lipids diacylglycerol and cardiolipin, dissolved in chloroform/methanol (1:1), were placed in 7-ml Schott glass disposable reaction tubes (with screw cap; Schott, Mainz, Germany), dried under a stream of nitrogen vapor, resuspended in a solution of sodium deoxycholate dissolved in water, and sonified for 5 min in a Sonorex RK 100 Sonifier (Bandelin, Berlin, Germany). The assay mix was preincubated for 5 min at 25 °C. ATP was added, and the reaction was stopped after 30 min by adding 750 μl of chloroform/methanol (1:2) containing 1% HCl. Extraction and separation of phosphoryliphids were performed as follows. 1 ml of chloroform/methanol (1:1) and 500 μl of 1 M KCl plus 0.2 M H3PO4 were added, and the mixture was mixed thoroughly on a vortexer; samples were centrifuged at 2000 rpm for 5 min in a Labofuge 200 (Heraeus Sepatech, Osterode, Germany). The lower phase (lipids) was transferred to a new glass reaction tube and dried under a stream of nitrogen vapor. Fifty μl of chloroform/methanol (2:1) were added to each tube, and the sample was applied to TLC silica plates (Merck) that had been treated with 1% potassium carbonate solution in methanol/water (2:3) and heated for 15 min at 110 °C. The plates were run with chloroform/acetone/methanol/ acetic acid/water (40:15:14:12:8). For autoradiography, the plates were exposed to x-ray film (Kodak X-Omat AR) for 1 day. The amount of phosphate incorporated was determined by scratching off the individual spots and counting the radioactivity in a liquid scintillation counter. PA standard was obtained from Sigma (product code P 8511). Amersham Biosciences provided [γ-32P]ATP (15 Tih/mmol). Lipids (1,2-SAG, 1,2-DOG, 1,2-DOC, 1,2-DPG, and cardiolipin), salts (MgCl2, CaCl2, LiCl, and NaCl), detergents (Na-DC and CHAPS), and the DGK inhibitor R59022 were purchased from Sigma. Serva (Heidelberg, Germany) provided Triton X-100.

HPLC Analysis of the Reaction Products of DGK Assay—The products of diacylglycerol kinase assays were obtained by two-phase partitioning. The organic phase was reduced to dryness under a stream of nitrogen gas, and the glycerolipids were deacylated (26). The water-soluble products of deacylation were resolved by anion exchange high performance liquid chromatography with a gradient of (NH4)2HPO4 (27). Radioactivity was monitored on-line with a Canberra Packard Aut15 Radiomatic Flow Detector, integration interval 12 s.

RESULTS

Isolation of AtDGK2 cDNA and Sequence Characterization—A partial cDNA encoding a polypeptide similar to AtDGK1 from A. thaliana and similar to DGKs from animals was retrieved from an expression profiling experiment that was targeted toward the identification of differentially expressed genes in leaf tissues. A cDNA encompassing the entire coding region of the novel DGK, designated AtDGK2 (for A. thaliana diacylglycerol kinase 2), was subsequently cloned via high fidelity polymerase chain reaction, using a pool of leaf first-strand cDNAs as template. The AtDGK2 protein deduced from the cDNA sequence has a molecular mass of 79.4 kDa (712 amino acid residues) and an isoelectric point of 8.45. The overall structure of AtDGK2 and of all other Arabidopsis DGKs (see below) is displayed in Fig. 1a. The AtDGK2 protein shares 50% identical amino acids with AtDGK1, 39% with human DGKα (GenBank™ accession number NM_001345), 28% with human DGKβ (NM_004717), 39% with Caenorhabditis elegans Dgk-1 (NM_075789), and 30% with C. elegans Dgk-3 (NM_066630). The closest other plant homologue (45% identical amino acids) is a predicted DGK from rice encoded by transcript AIK100331. All DGKs have a conserved catalytic domain, DGKc (Pfam accession number PF00781) (Fig. 1, a and b), which in AtDGK2 includes amino acid residues 342–471. DGKc domains contain a preserved ATP binding site with a GXGXXG consensus sequence (where G represents glycine, and X represents any amino acid) (28). Although the presumed ATP binding site in AtDGK2 (GI(401–Ala406)) has an alanine instead of a glycine residue at the end of this motif, it is obvious from our results (see below) that this change does not render the enzyme inactive. An uncharacterized AtDGK2 homologue from rice (GenBank™ accession number AK100331; see below) also contains a GXGXXA motif.

AtDGK2 also harbors a presumed DGK accessory domain (DGKa; Pfam accession no. PF00609), which is associated with DGKc, indicating that it contributes to the functionality of the catalytic domain. The accessory domain (158 aa) is located at residues 491–648. Furthermore, this enzyme contains two copies of about 60 amino acids each of a PKC, diacylglycerol/ phorbol ester-binding domain (DAG/PE-binding domain; InterPro accession number IPR002219) between residues 72–133 and 145–208, respectively. PE are analogues of DAG and poten- tient tumor promoters in animals. PE can directly stimulate PKC. PE and DAG bind in a zinc-dependent manner to the so-called C1 region of the protein (29). The C1 region contains one or two copies (depending on the PKC iso)zyme of a cysteine-rich domain (CRD), which is essential for DAG/PE-binding. C1 domains are also present in animal DGKs, where they exist in two or three copies, designated as C1A, C1B, and C1C. Recent studies using NMR spectroscopy and x-ray crystallography have clarified the three-dimensional structure of C1 domains of various PKC isoforms. The typical core structure of C1 domains in PKC contains two histidines and six cysteines in the order HX12CX2CX3H2–1, CX4CX5HX6CX6–1C (where X represents any amino acid), coordinating two zinc atoms in a tetra- hedrally geometry. The core of animal DGK C1 domains is slightly different, HX10–12CX2–6CX9–15 CX4CX5HX6–1CX4–10C, but the zinc-coordinating histidine and cysteine residues are present (29). We screened the GenBank™ data base using the AtDGK2 sequence as a bait and retrieved cDNA sequences from Oryza sativa (GenBank™ accession number AK100331) and Zea mays (partial cDNA, resulting from an EST assembly project; AY110054). Like AtDGK2, both monocot proteins contain two copies of a DAG/PE-binding domain (although one of each was not fully supported by either PROSITE or SMART; in the maize protein, this was due to the fact that the coding sequence close to the 5′ end of the EST-assembled cDNA was uncertain). To test for the existence of additional C6/H2-type DGKs, we screened the TAIR data base (available on the World Wide Web at www.arabidopsis.org/) using the Patmatch tool. None of the proteins retrieved (with the exceptions of AtDGK1 and AtDGK2) contained a DGK catalytic domain, indicating that no additional DGKs of the C6/H2-type are present in Arabidopsis.

DAG/PE-binding domain 1 has the core HX14CX2CX16–24 CX2CX2HX2CX2C in all four identified plant proteins (i.e.

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2 M. Ornatowska, M.-J. Zanor, G. Plesch, and B. Mueller-Roever, unpublished results.
AtDGK1 and AtDGK2, as well as the rice and maize enzymes), with a flexible length of the amino acid stretch after the second cysteine. In contrast, DAG/PE-binding domain 2 has the invariant core HX_{18}CX_{16}CX_{16}CX_{16}CX_{16}CX_{16}CX_{16}CX_{16}C (Fig. 1b). In animal DGKs, the more C-terminally located DAG/PE-binding domain contains a conserved 15-amino acid extension; this domain was therefore designated the "extended cysteine-rich domain" (exCRD) (30). We observed that a very similar amino acid extension also adjoins the second, but not the first, DAG/PE-binding domain of the plant DGKs (Fig. 1b), indicating evolutionary conservation. It was suggested that the exCRD, in conjunction with the conserved catalytic domain, is essential for the unique enzymatic function of DGK (30, 31). Recently, it was demonstrated that C1A domains in animal DGKs also contained basic amino acid segments, which are almost identical in sequence, upstream of DAG/PE-binding domain 1 (rice, YTVNQLKNNLSLMLKAIARARKYKKLDKVP; maize, YTVNQLKNNLSLMLIARAIKARAKYKKSKDKVP). The two monocot DGKs also contain basic amino acid segments, which are almost identical in sequence, upstream of DAG/PE-binding domain 1 (rice, YTVNQLKNNLSLMLKAIARARKYKKLDKVP; maize, YTVNQLKNNLSLMLIARAIKARAKYKKSKDKVP). In all four plant enzymes analyzed here, the distances between the upstream basic region and the first copy of the DAG/PE-binding domain (3 amino acids) and between the

![Structural organization of DGK isozymes from A. thaliana.](image)

**a** A schematic representation of the primary structure of AtDGK isozymes. **b** Details of the domain organization of plant Cluster I DGKs. Approximate positions and sequences of the conserved C_{6}/H_{2} cores are shown. Basic amino acid residues (Lys and Arg) in the upstream basic regions are indicated in boldface type. Conserved amino acid residues in the exCRD-like elongation are also shown in boldface type. An animal exCRD consensus sequence (30) is included for comparison. For accession numbers of Arabidopsis DGKs, refer to Table I; other numbers are as follows: rice, AK100331; maize, AY110054.

AtDGK2 and Related Plant DGK Isozymes Contain a Unique Aggregation of Basic Amino Acids Upstream of DAG/PE-binding Domain 1—AtDGK2 contains an accumulation of basic amino acids upstream of DAG/PE-binding domain 1, flanked by residues YT and VP (YTVNQLKNNLSLMLKAIARARKYKKLDKVP) (Fig. 1, a and b). A similar, previously unrecognized, aggregation of basic amino acids is present in the amino-terminal part of AtDGK1 (YTVNQLKNNLSLMLIARAIKARAKYKKSKDKVP). The two monocot DGKs also contain basic amino acid segments, which are almost identical in sequence, upstream of DAG/PE-binding domain 1 (rice, YTVNQLKNNLSLMLKAIARARKYKKLDKVP; maize, YTVNQLKNNLSLMLIARAIKARAKYKKSKDKVP). In all four plant enzymes analyzed here, the distances between the upstream basic region and the first copy of the DAG/PE-binding domain (3 amino acids) and between the
two copies of the DAG/PE-binding domains (12 amino acid residues), respectively, were strongly conserved. Therefore, plant enzymes belonging to this DGK family (referred to as Cluster I enzymes; see below) have the following generalized structure: (YT-upstream basic region-VP)+(3 aa)-(DAG/PE-BD-1)+(12 aa)-(DAG/PE-BD-2/extra CRD-like)-(130 aa)-(DGKe/DGKa domain) (see Fig. 1b). This highly conserved domain organization indicates that the upstream basic region acts in concert with the DAG/PE-binding domains to fulfill its biological function. One speculative possibility is that it functions as a pseudosubstrate, autoregulating DGK activity, similar to protein kinase C (32).

Plant DGKs Fall into Three Phylogenetically Distinct Clusters—Multicellular organisms like humans, C. elegans, or Drosophila melanogaster contain multiple DGK genes (33). To check whether multiple DGK genes also exist in Arabidopsis, we analyzed the public data bases for the presence of putative DGK coding sequences. The result is summarized in Table I. The unique domain organization observed for AtDGK1 and AtDGK2 was not present in any other protein encoded by the Arabidopsis genome. However, five additional gene loci were detected that could potentially code for DGK proteins. In the case of locus At2g20900 two alternatively spliced transcripts, represented by full-length cDNAs, were found. The protein coding information of these transcripts is identical for the major part of the protein, up to amino acid position 484. The protein encoded by transcript NM_120874 (here designated AtDGK5α) contains an additional 7 amino acids, whereas DGK encoded by transcript NM_179649 (AtDGK5β) contains 25 extra amino acids, 6 of which are basic (arginine and lysine). Generation of the AtDGK5β splice variant is also evident from serial analysis of gene expression (SAGE) experiments, deposited in the NCBI GEO expression data repository (GEO Data Set accession number GDS101). Importantly, splice variants have also been reported for LeDGK1 from tomatoes (17). Whereas LeDGK1 itself does not bind calmodulin, the LeCB-DGK protein, which is encoded by an alternatively spliced transcript, contains a calmodulin-binding domain at its very C terminus. This extra protein domain, as in AtDGK5β, contains an enrichment of basic amino acid residues (10 of 28 amino acids are arginine or lysine). In addition, AtDGK5 was found to be the closest homologue of LeDGK1 (Fig. 2). Based on these observations, we propose that AtDGK5 represents the orthologue of the tomato gene LeDGK1, possibly coding for calmodulin-binding and nonbinding isoforms. A detailed analysis of plant sequences related to AtDGK5/LeDGK1 so far did not point to the presence of splice variants in other plant species (data not shown). It therefore remains to be tested whether or not alternative splicing (and, hence, the presence of calmodulin-binding isoforms) is a general feature of genes orthologous to AtDGK5/LeDGK1.

The proteins encoded by the genes AtDGK3 to AtDGK7 have a predicted molecular mass of ~55 kDa (i.e. they are substantially smaller than AtDGK1 and AtDGK2, in part due to the lack of a typical DAG/PE-binding domain). The predicted DGK isoforms possess a catalytic (DGKe) and a presumed accessory domain (DGKa), similar to AtDGK1 and AtDGK2. No further known protein motifs could be identified in the small DGKs when scanned using the InterPro or Motif Scan interfaces (not shown).

To assess the phylogenetic relationship among plant DGK isoforms, sequences homologous to Arabidopsis DGK genes were retrieved from the GenBank™ data base. Proteins predicted from the sequences were aligned using ClustalX (34) and subjected to phylogenetic analysis. Only high quality sequences that unambiguously encoded plant DGKs were included in this analysis. As shown in Fig. 2, plant DGKs fall into three major clusters. Cluster I comprises the large (i.e. DAG/PE-binding site-containing) DGK isoforms, including AtDGK1 and AtDGK2 from Arabidopsis and the rice DGK encoded by transcript AK100331. Because no unambiguous amino acid sequence could be assigned to the Z. mays AY110054-encoded protein (see above), it was not included in the phylogenetic tree displayed in Fig. 2. However, manual inspection proved that the maize protein also belongs to Cluster I (not shown). Cluster II encompasses Arabidopsis isoforms AtDGK3, AtDGK4, and AtDGK7. Although Cluster II full-length sequences could not be retrieved from GenBank™ for any other plant species, it is evident from EST data analysis that Cluster II DGKs exist in many other plants, including, for example, Vitis vinifera (GenBank accession number CB981130), apricot (CB821694), tomato (AW035995), and Populus (BU828590) (data not shown). The isoforms AtDGK5 and AtDGK6 from Arabidopsis as well as additional DGK proteins from various other plant species constitute Cluster III. Taken together, representatives of all three DGK clusters are widely distributed in vascular plants. In addition, DGK-encoding ESTs were found in the moss Physcomitrella patens (AW126728 and BJ166785). The moss DGKs appeared to be more closely related to cluster II and III enzymes of vascular plants; however, due to fragmentary se-

### Table I

| Gene   | Locus | Accession number | Amino acid residues | Molecular mass (kDa) | cDNA/EST |
|--------|-------|------------------|---------------------|----------------------|----------|
| AtDGK1 | At5g07920 | NM_120874 | 728 | 80.0 | BT004148 |
| AtDGK2 | At5g38770 | NM_125772 | 712 | 79.4 | BT008792 |
| AtDGK3 | At2g18730 | NM_179649 | 488 | 53.9 | AF064982 |
| AtDGK4 | At5g57690 | NM_125152 | 498 | 55.5 | No cDNA/EST |
| AtDGK5 | At2g20900 | NM_127660 | 491 | 55.3 | ATDGK5α | 55.3 (AY085589), AF360300 |
| AtDGK6 | At4g28130 | NM_119853 | 509 | 57.4 | ATDGK5β | 57.4 (AY085589), AF360300 |
| AtDGK7 | At4g30340 | NM_119853 | 509 | 52.5 | No cDNA/EST |

**A** Gene names marked with an asterisk are the same as in Kanoh et al. (15); the other genes were not mentioned, or a wrong accession number was given.

**b** Accession numbers of the NCBI RefSeq database (available on the World Wide Web at www.ncbi.nlm.nih.gov/RefSeq/), with the exception of F17123.320, which is a locus name provided by TIGR (available on the World Wide Web at www.tigr.org/).

**c** GenBank™ accession numbers.

**d** The two cDNA clones represent splice variants of the At2g20900 locus.

**e** Two genes, At2g28120 and At2g28130 (GenBank™ accession no: AL161572) were originally predicted to reside closely linked on chromosome 2 and to encode polypeptides similar to diacylglycerol kinase. Later, the At2g28130 locus was re-annotated (NM_118953).

**f** Due to a lack of confirmed cDNA sequences, an unambiguous coding region could not yet be assembled.

**g** Annotated as unknown protein.
sequence information, unequivocal cluster assignment was not possible. In A. thaliana, a further gene (present at locus At2g46090) codes for an unknown protein featuring a potential DGKc domain in its N-terminal half. This protein, however, does not contain a DGKa domain, and it did not pick up any DGK sequence from plant or animal sources when used as a bait in various BLAST searches (not shown), indicating that it represents a distinctly related protein of a different biological function.

For most of the Arabidopsis DGK genes, cDNA or EST entries were deposited in the public data bases (Table I), demonstrating that members of each of the three DGK clusters are expressed. In addition, expression of AtDGK5 is evident from various microarray and SAGE experiments deposited in the NCBI GEO data base (accession numbers GDS5, 101, 135, 207, and 208). However, no cDNA/EST sequences could be retrieved from the public data bases for predicted genes AtDGK4 and AtDGK6, leaving the question open whether these genes are also transcribed. Furthermore, biochemical studies are required to demonstrate that the predicted proteins indeed code for enzymatically active DGKs, namely in the absence of a DAG/PE-binding domain. However, as mentioned before, earlier studies have shown that tomato LeDGK1, which lacks such a domain, exhibits diacylglycerol kinase activity upon heterologous expression in E. coli (17).

AtDGK2 Encodes a Functionally Active DAG Kinase That Phosphorylates 1-Stearoyl-2-arachidonoyl-sn-glycerol and 1,2-Dioleoyl-sn-glycerol—So far, enzymatic activity for plant DGKs has only been reported for LeDGK1 from tomato (17). No enzyme activity was demonstrated for any DAG/PE-binding domain-containing DGK from plants. We therefore tested recombinant polyhistidine-tagged AtDGK2, expressed in E. coli (see “Experimental Procedures” for details) for its potential to phosphorylate DAG. After isopropyl-β-D-thiogalactopyranoside induction, protein was extracted for both immunoassays and enzymatic analyses. Recombinant fusion protein of an apparent molecular mass of ~140 kDa was detected by an anti-His tag antibody in extracts of the transformed cells (Fig. 3, lane 1), which compares favorably with the predicted size of the fusion protein (AtDGK2, 79.4 kDa; NusA tag/His6 tag, ~60 kDa). Extracts from cells transformed with the pET43c control vector (lacking the AtDGK2 cDNA) reacted with the antibody, revealing a protein of ~60 kDa that corresponds to the NusA tag/His6 tag fusion expressed from the empty vector itself. We also fused the AtDGK2 cDNA to the His tag coding region in vector pET28c, which lacks the NusA tag fusion sequence. In this case, after isopropyl-β-D-thiogalactopyranoside induction, no immunoreactive His-tagged AtDGK2 fusion protein was detectable in Western blot experiments, even when experimental and growth conditions were varied (not shown), indicating absence of protein expression.

Purified proteins from cells transformed with the AtDGK2 cDNA or the empty vector (pET43c) were assayed for DGK enzymatic activity. Cells transformed with AtDGK2 showed strong DAG kinase activity, whereas the vector-only transformants exhibited no activity after autoradiography of TLC plates (Fig. 4, a and b), demonstrating that AtDGK2 encodes a functional lipid kinase. This result was confirmed by analyzing the products of deacylation resolved by anion exchange HPLC.
Arabidopsis Diacylglycerol Kinase AtDGK2

The enzyme showed a Michaelis-Menten-type saturation curve for the substrate 1,2-DG (Fig. 5b). Calculated $K_m$ and $V_{max}$ values (Fig. 5c) were 125 $\mu M$ for 1,2-DG and 0.25 pmol PA min$^{-1}$ $\mu g$ of protein$^{-1}$, respectively under these conditions. The $K_m$ of 125 $\mu M$ for 1,2-DG is rather low and indicates the high affinity of the enzyme for the substrate; for other DGK enzymes, $K_m$ values of up to 1 mM were reported (35, 36). Regarding the specific activity, values of 3–20 pmol PA min$^{-1}$ $\mu g$ of protein$^{-1}$ were previously reported in biochemical studies for wheat (37) and tomato (17) DGK isoforms, both being significantly higher than the specific activity observed here for Arabidopsis AtDGK2. A specific activity of 0.4 pmol of PA min$^{-1}$ $\mu g$ of protein$^{-1}$ has been reported for the human DGKz isoform (38), whereas another human DGK isoform, DGKx, showed values higher than 20 pmol of PA min$^{-1}$ $\mu g$ of protein$^{-1}$ (39).

The enzyme has an activity optimum at pH 7.2 and exhibits a fairly rapid decrease of activity below pH 6.5 and above pH 7.8 (Fig. 5d). AtDGK2 activity was tested in the presence of different types of detergents. CHAPS at 1–10 mM concentration proved to be the most efficient detergent for solubilization of lipids, whereas Na-DC at a concentration of already 5 mM and Triton X-100 at up to 2% drastically reduced AtDGK2 activity (Fig. 5e). The lack of an EF-hand in the enzyme structure suggests that calcium would not affect the activity to a large extent. No change of PA formation was observed when CaCl$_2$ (1–1000 $\mu M$) was added to the reaction mix (data not shown). NaCl and LiCl reduced the activity of the enzyme by more than 50% as compared with the standard assay, when the salt concentration was raised to more than 200 mM (Fig. 5f).

We have examined the substrate specificity of expressed AtDGK2 to inquire whether activity against DAG substrate analogs could illuminate cellular activity against other potential signaling substrates. Our data indicate that AtDGK2 has high specificity for 1,2-SAG and 1,2-DOG (Fig. 6a). Interestingly, 1,2-SAG and 1,2-DOG are more unsaturated (18:0/20:4 and 18:1/18:1 for 1,2-SAG and 1,2-DOG, respectively) than the other lipids tested. Similarly, in other studies with human DGKs, a strong preference for 1,2-SAG and 1,2-DOG was observed (28, 36). Saturated diacylglycerols (1,2-DCCO, 1,2-DPG, and 1,2-DMG) or monosaturated DAG analogs (1,2-OAG) were poor substrates for AtDGK2 (Fig. 6a).

To further confirm that the formation of PA was a result of AtDGK2 activity, the reaction mix was preincubated with the DGK inhibitor R59022, which has been shown to be active in both animal and plant cells (18, 37, 40, 41). In our experiments, the decrease in PA formation was dependent on the concentration of the inhibitor (Fig. 6b). The R59022 concentration needed for half-maximal inhibition ($IC_{50}$) was 50 $\mu M$. We observed that the enzyme was still partially active at 1 mM R59022 concentration.

Expression Pattern of the AtDGK2 Gene—We performed Northern blot experiments to test the expression pattern of the AtDGK2 gene. To this end, RNA was isolated from various organs of Arabidopsis plants grown in the greenhouse. As shown in Fig. 7a, AtDGK2 transcript was detected in rosette leaves, flowers, siliques, and roots. Expression of AtDGK2 was particularly strong in cauline leaves collected from the inflorescence stem. In contrast, AtDGK2 mRNA was undetectable under our assay conditions in the inflorescence stem itself (Fig. 7a), indicating that DGK is not required in this organ under normal growth conditions or, more likely, that another AtDGK gene provides DGK protein. AtDGK1 may be a candidate, since it has been reported to be transcribed in shoots (16). Promoter-reporter gene studies (described below) revealed that AtDGK2 expression is more prominent in young leaves, whereas it declines in older leaves. A similar result was obtained in Northern blot experiments. AtDGK2 mRNA was detectable in leaves throughout all developmental stages, but its abundance decreased in leaves when plants became older (Fig. 7b).
To assess the transcriptional activity of the AtDGK2 gene further, a 1.2-kb-long 5' upstream region of the AtDGK2 gene was isolated, fused to the E. coli β-glucuronidase (GUS) reporter gene in binary plasmid pCAMBIA-1303, and transformed into A. thaliana. The promoter was also inserted upstream of the GUS gene in vector pGPTV-HPT. Transgenic
plants were selected on axenic medium containing hygromycin, and more than 60 independent promAtDGK2::GUS transformants were obtained from both transformations. From these, more than 50 lines exhibited GUS activity when tested on the seedling stage. The GUS activity patterns were not distinguishable in the two sets of plants.

We first tested for the distribution of GUS activity throughout various stages of seedling development. Seeds from promAtDGK2::GUS lines were germinated on agar plates, and strong GUS activity was detected in 1-day-old seedlings, still being covered by the seed coat (Fig. 8a). On days 2 and 3, strongest GUS staining was observed at the root-shoot junction zone, whereas GUS activity was mostly absent from the root apex (Fig. 8b and c). In cotyledons, GUS staining was evenly distributed (Fig. 8d). From day 4 onwards, GUS staining became more distinct in the vascular bundles of the cotyledons (Fig. 8e–g). In these seedlings, AtDGK2 promoter activity also shifted toward the root tip, indicating a change in the physiological makeup of the root at this developmental stage (see Fig. 8k and l, for 15- and 7-day-old seedlings, respectively). In roots from older plants, AtDGK2 activity was mostly concentrated in the central cylinder (Fig. 8m). Root hairs also exhibited GUS activity (Fig. 8h and i). The preference for GUS staining in vascular tissue remained at later stages of plant development when plants were grown in soil (see Fig. 8k). However, mesophyll cells and guard cells were also stained (Fig. 8, n–p), especially in young leaves. Generally, GUS activity was more pronounced in young and cauline leaves (Figs. 8, n–p). In flowers, prominent GUS staining was detected in sepalts, filaments of the anthers, and the stigma (Fig. 8q). GUS activity

![Substrate specificity of the enzyme AtDGK2 and inhibition by R59022.](image)

**FIG. 6.** Substrate specificity of the enzyme AtDGK2 and inhibition by R59022. a, AtDGK2 activity was assayed with various DAG analogs. For each substrate, the concentration used in the reaction mix was 500 μM. Values are expressed as percentage of enzyme activity determined under control assay conditions using 500 μM 1,2-DOG. Values are means of three independent determinations ± S.D. b, R59022, dissolved in dimethyl sulfoxide (DMSO), was added prior to the start of the reaction. Values are expressed as percentages of enzyme activity determined under control assay conditions (i.e. in the absence of R59022) (means of three independent determinations ± S.D.).

![Organ-specific expression of AtDGK2.](image)

**FIG. 7.** Organ-specific expression of AtDGK2. a, Northern blot analysis of AtDGK2 transcript levels in different organs of A. thaliana plants grown in soil. b, AtDGK2 transcript level in rosette leaves of different developmental stages. Blots were hybridized to radiolabeled AtDGK2 cDNA. Upper panels in a and b, hybridization signals visualized by autoradiography; lower panels, ethidium bromide-stained RNA gels (loading controls).
was also observed in the valves of young and early adult silique tissues at the tip. Adult silique showing strongest GUS staining in valves and stigmatic tissue at the tip. Note strong GUS staining at the root tips (arrows) shown are roots (a) and root hairs (i) of a 7-day-old seedling. k, 15-day-old seedling. Note strong GUS staining at the root tips (arrows), which is also seen in l for a 7-day-old seedling. m, cross-section through a root of a 6-week-old plant grown in soil. GUS activity is concentrated in the central cylinder. n, strong GUS staining is visible in cauline leaves but not the inflorescence stem. o, GUS staining is maximal in young leaves (left) but decreases when leaves become older (right). p, mesophyll cells (mc) and guard cells (gc), released by applying gentle pressure to a young leaf kept on a glass slide, exhibit GUS staining. q, mature flower with intense GUS staining of sepals, filaments, and the stigma. r, in seeds, strong GUS staining is visible at the hilum (arrow). s, an early adult silique showing strongest GUS staining in valves and stigmatic tissue at the tip.

Fig. 8. Analysis of GUS activity in transgenic Arabidopsis plants transformed with the promAtDGK2::GUS fusion construct. a, seed kept for 1 day on agar medium. Note GUS staining of the embryo inside the seed. b and c, 2-day-old seedlings. GUS staining is absent from the root apex (arrows). d, 3-day-old seedling. e, 4-day-old seedling. f, 5-day-old seedling. g, cotyledon of a 5-day-old seedling. Also shown are roots (h) and root hairs (i) of a 7-day-old seedling. k, 15-day-old seedling. Note strong GUS staining at the root tips (arrows), which is also seen in l for a 7-day-old seedling. m, cross-section through a root of a 6-week-old plant grown in soil. GUS activity is concentrated in the central cylinder. n, strong GUS staining is visible in cauline leaves but not the inflorescence stem. o, GUS staining is maximal in young leaves (left) but decreases when leaves become older (right). p, mesophyll cells (mc) and guard cells (gc), released by applying gentle pressure to a young leaf kept on a glass slide, exhibit GUS staining. q, mature flower with intense GUS staining of sepals, filaments, and the stigma. r, in seeds, strong GUS staining is visible at the hilum (arrow). s, an early adult silique showing strongest GUS staining in valves and stigmatic tissue at the tip.

pointing to a post-transcriptional mechanism of DGK activation. However, there is evidence in plants that genes for signaling proteins are often up-regulated by the stress for which they signal (42). Similarly, we observed that AtDGK2 transcript accumulates rapidly, within 30 min, after exposure to 4°C and, enhanced expression remained for up to 24 h (Fig. 9b). Analysis of the AtDGK2 promoter for the presence of potential cis regulatory elements using the PLACE (available on the World Wide Web at www.dna.aflrc.go.jp/htdocs/PLACE/fasta.html) and PlantCARE (available on the World Wide Web at intra.psb.ugent.be:8080/PlantCARE) interfaces revealed cis elements that may contribute to cold-responsive gene expression (Fig. 9a) (43–46). We tested the transcriptional activation of AtDGK2 by subjecting promAtDGK2::GUS reporter lines to cold stress. Transgenic plants from more than 10 independent transformants carrying the promoter-reporter gene construct were grown for 6 weeks and then subjected to cold treatment (4°C) for 12 h. Fig. 9c shows cold-enhanced GUS staining in the whole plant. Enhanced AtDGK2-driven GUS expression was already detectable after 1 h of cold treatment (Fig. 9d). In contrast, we could not obtain reproducible evidence for regulation of AtDGK2 expression by abscisic acid (100 μM, up to 5 h of treatment) or drought stress (data not shown).
DISCUSSION

In the present paper, we report the molecular cloning and characterization of a new diacylglycerol kinase from *A. thaliana*. *AtDGK2* belongs to a family of seven DGK genes in this plant. Phylogenetic analysis revealed that plant DGKs fall into three distinct clusters (Fig. 2). Members of all three clusters are widely distributed in the plant kingdom. Cluster I DGKs typically contain a DAG/PE-binding domain, which is flanked by an upstream basic region preceding DAG/PE-binding domain 1, and an extCRD-like sequence following DAG/PE-binding domain 2. The two copies of the DAG/PE-binding domain contain C/Hβ-type cores of slightly different amino acid arrangements (Fig. 1b), which also deviate from the C/Hβ core sequences of animal DGKs. Plant enzymes of Clusters II and III are simpler in organization, lacking the basic upstream region, the two DAG/PE-binding domains, and the extCRD-like motif, indicating that the special arrangement of the domains in Cluster I enzymes is essential for their function. The presence of a putative binding site for phorbol esters in Cluster I DGKs is interesting in light of the fact that phorbol esters are known to induce various cellular responses in plants. Thus, it has been observed that phorbol 12-myristate 13-acetate (PMA) alters the expression level of genes. For example, in tobacco suspension cells, PMA induces the accumulation of the defense gene *hsp203.1*, an effect that may be mediated by active oxygen species that transiently accumulate upon PMA treatment (47). PMA also stimulates transcription of the spinach *PsaF* gene, which encodes subunit III of the photosystem I reaction center (48, 49) and enhances the transcript level of nitrate reductase in the dark (50). PMA can also exert its effect on a post-translational level; a 45-kDa protein kinase was transiently activated in tobacco suspension cells in the presence of cycloheximide, indicating that protein synthesis was not required for the activation of the kinase (51). Physiological studies have shown that PMA can also trigger developmental processes (e.g. the initiation of adventitious buds in *Torenia fournieri* stem segments through the induction of meristemetic divisions in the epidermis (52). Although these experiments revealed that PMA affects plant cell biology, its mode of action remains elusive. In animal cells, PKC is a major target of phorbol esters; however, several nonkinase phorbol ester receptors have also been described (4). A gene for PKC has not been identified in plants. Some of the effects observed upon PMA application in plants may result from an interaction of phorbol esters with proteins other than PKC. Cluster I DGKs, such as AtDGK1 and AtDGK2, could be potential phorbol ester effectors in plants. Phorbol ester binding has recently been demonstrated for DGKα and DGKβ from animals (29). Whether plant DGKs also bind phorbol esters awaits further biochemical analysis. To our knowledge, AtDGK2 represents the only cloned DAG/PE-binding domain-containing plant DGK for which enzymatic activity has been demonstrated. The only other plant enzyme that exhibited DGK activity was previously cloned from tomato (17). The tomato enzyme lacks a typical DAG/PE-binding domain, indicating that the DAG/PE-binding domain is not essential for DGK activity.

The product of the DGK enzymatic reaction, PA, accumulates within minutes after a wide variety of stress treatments, including the application of the stress hormone abscisic acid, wounding, pathogen attack, or osmotic or oxidative stress (8), suggesting a role for PA as a general stress-signaling molecule. Recently, it has also been demonstrated that a cold (0 °C) treatment leads to an accumulation of PA. The major proportion (~80%) of the cold-induced PA was synthesized through the phosphoinositide-specific phospholipase C/DGK pathway, whereas PLD contributed only to a small extent (~20%) to PA formation (18). The involvement of DGK in this response was confirmed by performing experiments where cells were preincubated with the DGK inhibitor R59022 prior to the cold exposure, causing a severe reduction of PA formation at a concentration of around 50–100 μM of the inhibitor (18). We showed that AtDGK2 activity is also affected by R59022 with an IC₅₀ of 50 μM, suggesting that it is involved in the cold response previously reported (18). The observed PA response was rapid, occurring over the first 10 min of cold treatment, which makes it unlikely that gene activation is required, at least in the initial phase of the response. The observed induction of AtDGK2 gene expression may in part add to a sustained enhancement of PA concentration under cold stress, which, however, does not fully conform with the results reported by Ruelland et al. (18), who observed a decrease of PA content after prolonged cold treatment. On the other hand, cold induction was also reported for AtPLC1, which encodes a phospholipase C isoform in *A. thaliana* (20). Our data show that AtDGK2 is expressed in a variety of tissues. We noticed that expression was generally more pronounced in young tissues and along the vascular strands. The preference of expression for particular tissue types (and the change of expression in the root tip during early plant growth; see “Results”) may indicate tissue-specific functions of AtDGK2 in relation to plant growth.

Recombinant AtDGK2 activity was sensitive to the pH in the reaction mix and was affected by the presence of Mg²⁺, detergents, salts, and the inhibitor R59022. The formation of PA was inhibited by elevated levels of the anionic detergent Na-DC and the nonionic detergent Triton X-100. On the contrary, the zwitterionic detergent CHAPS (up to 10 mM) markedly stimulated AtDGK2 enzyme activity, apparently because it is more efficient at solubilizing both lipids (diacylglycerol and cardiolipin) and the recombinant protein, without disrupting the native state of the enzyme. Mixtures of diacylglycerol with cofactors such as diphosphatidylglycerol (cardiolipin) may facilitate the availability of DAG to the membrane-associated enzyme and together with the solubilizing effect of detergents may improve the enzymatic reaction. AtDGK2 activity was not significantly affected by CaCl₂ in the concentration range of 1–1000 μM under our assay conditions, indicating that AtDGK2 may not be regulated by cytoplasmic Ca²⁺ in vivo. Similar results were reported for tobacco and wheat DGKs (37, 53). Sodium chloride (up to 0.8 M) has been reported to inhibit DGK activity in *Rattus norvegicus* (54). Here we found that both NaCl and LiCl reduced DGK activity at lower concentrations. The IC₅₀ was around 200 mM for both salts.

Concerning the saturation of the acyl chains in the DAG analogs analyzed, we found that DAGs containing unsaturated acyl chains were, in general, better substrates for enzyme activity when they were incorporated into Na-DC micelles. In another study where the substrate specificity of human DGKβ was tested, a slight preference for 1,2-SAG was observed among the long chain DAGs examined (28).

In summary, the demonstration of activity for recombinant AtDGK isoform(s) of a class harboring DAG/PE-binding domains adds considerably to our scant knowledge of the molecular genetic basis of plant PA signaling. If PA proves to occupy center stage in plant lipid signaling, molecular analysis of the mechanisms of regulation of AtDGK isoform(s) will help reveal the functional significance of these proteins in plants and of DAG/PE-binding-domains across kingdoms. Gene knockouts and ectopic expressions will further such functional approaches.

Acknowledgments—We are very grateful to Dr. Amélie Kelly (MPI of Molecular Plant Physiology, Golm) and Dr. Stephan Elge (Free University, Berlin) for support during some of the biochemical experiments.
We thank the Max-Planck Institute of Molecular Plant Physiology, Golm, for providing laboratory space and infrastructure.

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